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(57) Abstract

A nucleotide molecule encoding a protein encoded by a Fos regulated gene or a fragment thereof, wherein said protein or fragment thereof is encoded by any one of the nucleotide sequences shown in Figure 1 or 2 or a fragment thereof, including allelic variants and species variants of the nucleotide sequences.

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REGULATED GENES AND USES THEREOF

The present invention relates to the nucleotide sequences of Fos regulated genes, the proteins encoded by the sequences, uses of the sequences and encoded proteins, and transgenic animals comprising one or more of the sequences. The present invention also relates to antibody molecules having affinity for the encoded proteins and uses of the antibody molecules, and antisense nucleotide molecules and uses of the antisense nucleotide molecules.

The transcription factor AP-1 is involved in a number cellular processes, including cell proliferation, differentiation and neuronal function (see Angel and Karin, 1991).

AP-1 is considered to exert its effect by binding to a DNA recognition sequence, known as the AP-1 element, found in the promoter and enhancer regions of genes. The AP-1 element has the consensus sequence in TGA G/C TCA.

A number of genes have been found which contain AP-1 elements in their regulatory regions including c-Jun (Angel *et al.*, 1988), MCP-1 (Rollins *et al.*, 1988), Stromelysin (Kerr *et al.*, 1988), Type I collagenase (Schonthal *et al.*, 1988) and Interleukin II (Farrar *et al.*, 1989).

AP-1 is composed of dimeric complexes formed between Jun (c-Jun, Jun-B and Jun D) and Fos (c-Fos, Fos B, Fra-1 and Fra-2) proteins. The Fos component of AP-1 has been found to be the limiting component of AP-1 activity in cycling cells (see Kovary and Bravo, 1991).

c-Fos is a nuclear proto-oncogene which has been implicated in a number of important cellular events, including a cell proliferation (Holt *et al.*, 1986; Riabowol *et al.*, 1988), differentiation (Distel *et al.*, 1987; Lord *et al.*, 1993 and tumorigenesis (Curren *et al.*, 1983; Miller *et al.*, 1984;

Ruther *et al.*, 1989).

c-Fos encodes a 62kDa protein which forms heterodimers with c-Jun, forming an AP-1 transcription factor which binds to DNA at an AP-1 element and stimulates transcription.

Fos gene products can also repress gene expression. Sassone *et al.* (1988) showed c-Fos inhibits its own promoter and Gius *et al.* (1990) and Hay *et al.* (1989) showed c-Fos inhibits early response genes Egr-1 and c-myc.

AP-1 factors have also been shown to inhibit expression of the MHC class I and PEPCK genes (see Gurney *et al.*, 1992 and Howcroft *et al.*, 1993).

It can therefore be seen that Fos regulated genes are extremely important for the correct expression of genes which lead to changes in the cell phenotype. The importance of Fos genes was clearly demonstrated by generating mice deficient in c-Fos (see Hu *et al.*, 1994). The c-Fos deficient mice were viable, but displayed a range of tissue-specific developmental defects, including osteopetrosis, delayed gametogenesis and lymphophenia and behaviourial abnormalities.

The c-Fos deficient mice were used to generate fibroblast cell lines and the expression of two genes was found to be abnormally low. The two genes were Stromelysin and Type I collagenase. Both genes were previously identified as having AP-1 sites in their regulatory sequences (see Kerr *et al.*, 1988 and Schonthal *et al.*, 1988).

Stromelysin and Type I collagenase have been implicated in embryonic tissue development (Brenner *et al.*, 1989), injured tissue remodelling (Hasty *et al.*, 1990; Woessner and Gunja, 1991) and in tumour progression and metastasis (Liotta and Stetler, 1990).

Superti-Furga *et al.*, (1991) showed that c-Fos activity can be controlled hormonally by fusing the mouse c-Fos protein to the ligand binding domain of the human estrogen receptor. The fusion protein was found to stimulate AP-1 dependant transcription in a strictly hormone-dependant manner. Using the fusion protein an AP-1 regulated gene, Fit-1, was found. Fit-1 was found to encode a secreted or membrane bound protein depending on the splicing pattern.

The present invention relates to the nucleotide sequences encoding two novel Fos regulated genes.

The present invention provides a nucleotide molecule encoding a protein encoded by a Fos regulated gene or a fragment thereof, wherein said protein or fragment thereof is encoded by a nucleotide sequence shown in Figure 1 or 2, or a fragment thereof, including allelic variants and species variants of the nucleotide sequences.

The term "nucleotide molecule" used herein refers to nucleotides of any length, either ribonucleotides or deoxyribonucleotides. The term encompasses both double and single stranded molecules. It also includes known types of modifications, for example labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those containing intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators and those containing modified linkages (e.g., alpha anomeric nucleic acids, etc.).

The nucleotide molecule of the present invention may encode the protein of a Fos regulated gene or a fragment thereof.

The term "fragment" used in relation to the proteins refers to fragments which are of sufficient length to be unique to the presently claimed protein (e.g., 10, 15, 20 or 25 consecutive amino acids in length). Preferably, the protein fragments are capable of eliciting at least part of an activity of the full protein. Particularly preferred fragments comprise a conserved region of a gene which has been found to be homologous with a number of other genes. Such conserved regions are considered to have a specific function.

The nucleotide sequences shown in Figures 1 and 2 will, as with most naturally occurring nucleotide sequences, have a number of other forms, such as allelic variants and species variants. Such variants and any other naturally occurring forms of the nucleotide sequences of the present invention are also considered to form a part of the present invention. Such variants should have at least 60%, preferably 80% and most preferably 90% sequence homology with the sequences shown in figure 1 or 2 or fragments thereof.

The present invention also relates to the nucleotide molecule of the present invention wherein the protein or a fragment thereof encoded by the sequence shown in Figure 1 or 2 or a fragment thereof is altered.

Preferred altered proteins or fragments thereof, are those that still retain their activity and preferably have a homology of at least 80%, more preferably 90% and most preferably 95% to the protein or a fragment thereof encoded by the sequence shown in Figure 1 or 2 or a fragment thereof. Preferably such altered proteins or fragments thereof differ by only 1 to 10 amino acids. It is further preferred that the amino acid changes are conservative.

Conservative changes are those that replace one amino acid with one from the family of amino acids which are related in their side chains. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid will not have a major effect on the biological activity of the protein.

However, it is sometimes desirable to alter amino acids in order to alter the biological activity of the protein. For example, mutations which abolish or enhance one or more of the functions of the protein can be particularly useful. Such mutations can generally be made by altering any conserved sequences of protein. Mutations which increase the number of amino acids which are capable of forming disulphide bonds with other amino acids in the protein are particularly preferred in order to increase the stability of the protein. Mutations which decrease the number of amino acids which are capable of forming disulphide bonds with other amino acids in the protein may also be made if it is desired to decrease the stability of the protein. It is preferred that such altered proteins or fragments thereof have a homology of at least 80%, more preferably 90% and most preferably 95% to the protein or a fragment thereof encoded by the sequence shown in Figure 1 or 2 or a fragment thereof. Preferably such altered proteins or fragments thereof differ by only 1 to 10 amino acids.

The nucleotide molecule of the present invention can be obtained by methods well known in the art. For example, the sequences may be obtained by genomic cloning or cDNA cloning from suitable cell lines or from DNA or cDNA derived directly from the tissues of an organism, such as a mouse. Suitable cell lines include any fibroblast cell lines such as the 3T3 cell line, described by Hu *et al.*, (1994). Positive clones may be screened using appropriate probes for the nucleotide molecule desired. PCR cloning may also be

used. The probes and primers can be easily generated given that the sequences encoding the protein or a fragment thereof encoded by the nucleotide molecule of the present invention are given herein.

Numerous standard techniques known in the field of molecular biology may be used to prepare the desired nucleotide molecules or the probes and primers for identifying the positive clones. The nucleotide molecules probes or primers may be synthesised completely using standard oligonucleotide synthesis methods, such as the phosphoramidite method.

Numerous techniques may be used to alter the DNA sequence obtained by the synthesis or cloning procedures, and such techniques are well known to those skilled in the art. For example, site directed metagenesis, oligonucleotide directed mutagenesis and PCR techniques may be used to alter the DNA sequence. Such techniques are well known to those skilled in the art and are described in the vast body of literature known to those skilled in the art, for example Sambrook *et al.*, (1989).

The present invention further provides the protein encoded by the nucleotide molecule of the present invention.

Preferably, the protein encoded by the nucleotide molecule of the present invention has the amino acid sequence shown in Figure 1 or 2, or a fragment thereof.

The term "protein" as used herein refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides and proteins are included within the term protein. The term also does not refer to or exclude post-expression modifications of the protein, for example, glycosylations, acetylations and phosphorylations. Included in the definition are proteins containing one or more analogs of an amino acid (including for example, unnatural amino acids), proteins with

substituted linkages, as well as other modifications known in the art, both naturally occurring and synthesised.

The protein of the present invention can be obtained from cells that naturally produce the protein such as fibroblast cells using standard purification techniques. However, it is preferred that a suitable host cell and vector system is used for the expression of the nucleotide molecule of the present invention. The nucleotide molecule of the present invention can be expressed in a variety of different expression systems, for example, those used with mammalian cells, baculoviruses, bacteria and eukaryotic microorganisms such as yeasts.

All the above mentioned expression systems are known in the art and expressing nucleotide sequences is now a standard technique known all skilled in the art.

Preferably, eukaryotic, e.g. mammalian, host cell expression systems are used. In particular, suitable mammalian host cells include chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BKH) cells, cells of hepatic origin such as HepG2 cells, and myeloma or hybridoma cell lines.

The present invention further provides a vector for the expression of the nucleotide molecule of the present invention, comprising a promoter and the nucleotide molecule of the present invention.

A mammalian promoter can be any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream transcription of a coding sequence into mRNA. Particularly useful promoters are those derived from mammalian viral genes, such as the SV40 early promoter, adenovirus major late promoter and the herpes simplex virus promoter. Additionally, sequences from non-viral genes can also be used as promoters, such as from the murine metallotheionein gene.

The nucleotide molecule of the present invention may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the nucleotide molecule of the present invention, in which case the first amino acid at the N-terminus of the encoded protein will be a methionine encoded by the start ATG codon.

Alternatively, the protein encoded by the nucleotide molecule of the present invention can be secreted from the cell by linking a nucleotide sequence encoding a leader sequence to the nucleotide molecule of the present invention. The encoded fusion protein will comprise a leader sequence fragment and the protein encoded by the nucleotide molecule of the present invention. The leader sequence will lead to the secretion of the fusion protein out of the cell. Preferably, there are processing sites between the leader sequence and the protein encoded by the nucleotide molecule of the present invention allowing the leader sequence to be cleaved off enzymatically or chemically. An example of such a leader sequence is the adenovirus triparite leader.

The vector of the present invention is preferably a nucleic acid vector comprising DNA. The vector may be of linear or circular configuration and can be adapted for episomal or integrated existence in the host cell, as set out in the extensive body of literature known to those skilled in the art. The vectors may be delivered to cells using viral or non-viral delivery systems. The choice of delivery system will determine whether the DNA molecule is to be incorporated into the cell genome or remain episomal.

The vector of the present invention can comprise further control elements such as polyadenylation signals, transcription termination signals, enhancers, locus control regions (LCRs), etc.

The present invention further provides a host cell transformed with the vector of the present invention.

Transformation refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector (episome), or may be integrated into the host genome.

Preferably, the host cell is a eukaryotic cell, more preferably a mammalian cell, such as chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BKH) cells, cells of hepatic origin such as HepG2 cells, and myeloma or hybridoma cell lines.

The present invention further provides a method for producing the protein encoded by the nucleotide molecule of the present invention, comprising transfecting a host cell with the vector of the present invention, culturing the transfected host cell under suitable conditions in order to lead to the expression of the DNA molecule and the production of the desired protein. The protein may then be harvested from the transfected cells or from the cell growth media, depending on whether the protein is secreted, using standard techniques.

The present invention further provides the nucleotide molecule of the present invention for use in therapy.

The present invention further provides the use of the nucleotide molecule of the present invention in the manufacture of a composition for the treatment of developmental disorders.

The present invention further provides the use of the nucleotide molecule of the present invention in the treatment of developmental disorders.

Fos regulated genes are known to be involved in development and cell differentiation. Accordingly, the discovery of genes regulated by Fos has implications in the control of development and cell differentiation.

The nucleotide sequences shown in Figure 1 and Figure 2 have been found to have a similar sequence to genes of a family of growth factors characterised by the Platelet Growth Factor (PDGF) family signature. The most clearly related sequence is that of the Vascular Endothelial Growth Factor (VEGF). VEGF forms a homodimer which is a growth factor active in angiogenesis and endothelial cell growth (see Keck *et al.*, 1989 and Leung *et al.*, 1989). VEGF has also been used to stimulate angiogenesis and thereby produce a therapeutic effect (see Takeshita *et al.*, 1994).

The protein encoded by the sequence in Figure 1 is a mouse protein and the protein encoded by the protein in Figure 2 is the human homologue of the mouse protein encoded by the sequence given in Figure 1. Both the proteins are herein refered to as c-Fos Induced Growth Factor (FIGF).

The use of the nucleotide molecule of the present invention in therapy can therefore be seen to be an important application of the sequences of the Fos regulated genes of the present invention.

The nucleotide sequences shown in Figure 1 and Figure 2 are of particular interest in lung disorders as it is has been found that the nucleotide sequences are mainly expressed in the lungs. Particular lung disorders which may be treatable using the nucleotide molecule encoding the protein or fragments thereof which are encoded by the sequence shown in Figure 1 or Figure 2, include pneumonia and pneumoconiosis. The nucleotide molecule may also be used following pneumonectomy in order to aid in lung re-growth.

The nucleotide sequence in Figure 2 has been mapped to human chromosome Xp22, near the locus that maps for the pathology spondyloepiphyseal dysplasia (SEDL). The genetic map of this region is described by Ferrero *et al.* (1995) and the mapping of the SEDL disease is described by Heuertz *et al.* (1993). SEDL may therefore be treatable using the nucleotide molecule encoding the protein or fragments thereof, which are encoded by the sequence given in Figure 1 or in Figure 2.

As previously discussed, Fos regulated genes have been found to be involved in tumour progression and metastasis. By inhibiting Fos regulated genes it is possible to inhibit or suppress tumour growth.

Previously Kim *et al.*, (1983) suppressed tumour growth by injecting monoclonal antibodies specific for VEGF. As stated previously, VEGF has a similar nucleotide sequence to the nucleotide sequences shown in Figure 1 and Figure 2. Accordingly, by inhibiting either the *in vivo* expression, translation, etc. of the native nucleotide molecules of the present invention, tumour growth may be inhibited or suppressed.

The actions of the Fos regulated genes corresponding to the nucleotide molecules of the present invention may be inhibited by a number of techniques. Preferred techniques include antisense based techniques, ribozyme based techniques, and antibody based techniques.

Antibody molecules having specificity for the protein encoded by the nucleotide molecules of the present invention can be used to block the function of the protein and thereby inhibit or suppress tumour growth.

The present invention further provides antibody molecules having specificity for the protein of the present invention.

The antibody molecules may be a complete polyclonal or monoclonal antibody or antigen binding fragments, such as Fv, Fab, $F(ab')_2$ fragments and single chain Fv fragments thereof. The antibody molecule may be a recombinant antibody molecule such as a chimeric antibody molecule preferably having human constant regions and mouse variable regions, a humanised CDR grafted antibody molecule or fragments thereof. Methods for producing such antibodies are well known to those skilled in the art and are described in EP-A-0120694 and EP-A-0125023.

The present invention further provides the antibody molecule of the present invention for use in therapy.

The present invention also provides the use of the antibody molecule of the present invention in the manufacture of a composition for the treatment of proliferative diseases such as cancer.

The present invention further provides the use of the antibody molecule of the present invention for the treatment of proliferative diseases such as cancer.

The present invention further provides an antisense nucleotide molecule or a fragment thereof, having the complementary sequence to the nucleotide molecule or a fragment thereof, of the present invention.

The antisense nucleotide molecule of the present invention can be generated using the same standard techniques as for the nucleotide molecule of the present invention.

The present invention further provides an antisense vector for the expression of the antisense nucleotide molecule of the present invention comprising a promoter and the antisense nucleotide molecule.

The antisense vector is identical to the nucleic acid vector

of the present invention except that the vector contains the antisense nucleotide molecule of the present invention.

The present invention further provides the antisense vector of the present invention for use in therapy.

The present invention further provides the use of the antisense vector of the present invention in the manufacture of a composition for the treatment of cell proliferative diseases such as cancer.

The present invention further provides the use of the antisense vector of the present invention in the treatment of cell proliferative diseases such as cancer.

The present invention further provides a vector for the expression of a ribozyme, comprising a promoter and a nucleotide sequence encoding a ribozyme capable of cleaving the RNA transcript of the nucleotide molecule of the present invention.

The vector encoding the ribozyme is identical to the vectors previously described except that the vector encodes a ribozyme. The ribozyme being capable of cleaving the RNA transcript of the nucleotide molecule of the present invention. Techniques for producing such ribozymes are known to those skilled in the art and are discussed in Cantor *et al.*, (1993).

The present invention further provides the ribozyme encoding vector of the present invention for use in therapy.

The present invention further provides the use of the ribozyme encoding vector of the present invention in the manufacture of a composition for the treatment of cell proliferative diseases such as cancer.

The present invention further provides the use of the

ribozyme encoding vector of the present invention in the treatment of cell proliferative diseases such as cancer.

A further object to the present invention is the use of the protein of the present invention in identifying the receptor or receptors of the protein or of a protein complex comprising the protein.

Methods for identifying receptors are well known to those skilled in the art and have been widely described in the literature. However, basically there are three major ways of identifying receptors:

i. Test all known receptors that bind to similar molecules. This is particularly useful for the protein encoded by the DNA sequences shown in Figure 1 and Figure 2, as VEGF has been found to have a similar sequence.

ii. Perform a binding purification step. For example, the protein of the present invention or a protein complex comprising the protein of the present invention can be immobilised on to a solid support and numerous possible receptor molecules, especially membrane proteins, are then passed over the solid support. A binding purification procedures is described in Schusted *et al.*, (1995).

iii. By screening expression libraries in order to find cells lacking the receptor or receptors and then utilising the receptor cloning method described by Seed and Aruffo, (1987).

Other methods are also known to those skilled in the art and can be used in order to find the receptor or receptors.

On identifying the receptor or receptors it will be possible to design drugs that block or enhance the activity of the receptor or receptors and produce antibody molecules that

block the receptor or receptors. Once the DNA sequence of the receptor or receptors are known, a number of gene therapies may be designed for correcting errors in the receptor or receptors, or for blocking expression of the receptor or receptors.

The present invention further provides the use of the protein of the present invention in an assay for identifying antagonists or agonists of the protein which may be used as drugs in the treatment of cancer and developmental disorders respectively. Assays for identifying such potential drugs are frequently used and are well known to those skilled in the art. An example of such an assay is clearly described in Tsunoda *et al.*, (1994).

The present invention further provides the use of the nucleotide molecule, antisense nucleotide molecule, protein or antibody molecule of the present invention or any combination thereof, in diagnosing a pathological state or a predisposition to a disease.

The nucleotide molecule or antisense nucleotide molecule of the present invention may be used in determining the presence of the gene corresponding to the nucleotide molecule or in determining the amount of RNA transcribed from the gene.

The protein of the present invention may be used in an assay for determining the amount of protein encoded by the gene corresponding to the nucleotide molecule of the present invention.

The antibody molecule of the present invention may be used in an assay for determining the amount of protein encoded by the gene corresponding to the nucleotide molecule of the present invention. An example of an assay for determining the amount of protein using the antibody molecule of the present invention is a competitive binding assay.

By determining the presence of the gene corresponding to the nucleotide molecule of the present invention or the transcribed RNA or the protein encoded by the gene, it is possible to diagnose a pathological state or a predisposition to a disease caused by the presence of the gene of the over expression of the gene.

The present invention further provides the use of the nucleotide molecule of a present invention in the generation of transgenic animal. In particular, the invention provides the use of such nucleotide molecules for the generation of non-human transgenic animals, especially transgenic mice.

Transgenic animals can be generated which are suitable as models for research. For example, transgenic animals which overexpress the nucleotide molecule of the present invention could be used in order to determine what effects overexpression will have. Alternatively, transgenic animals can be generated where the native nucleotide molecule of the present invention is "knocked out". The effect of "knocking out" the nucleotide molecule could then be investigated.

Methods for generating such transgenic animals are well known to those skilled in the art and can be easily performed given that the nucleotide molecules to be over expressed or "knocked out" are disclosed herein.

The transgenic animals of the present invention could also be subsequently bred with either Fos over expression mice or Fos "knocked out" mice, in order to determine the effects of altered Fos control.

The present invention also provides a nucleotide molecule comprising all or part of the sequence shown in any one of Figures 1 or 2.

The nucleotide molecule comprising all or part of the

sequence shown in any one of Figures 1 or 2 may encode a protein or may be non-coding. Preferably, the nucleotide molecule additionally encodes the control sequences of the Fos gene corresponding to the nucleotide sequence shown in any one of Figures 1 or 2. It is further preferred that the nucleotide molecule encodes a sequence which confers Fos regulation to a gene. It is particularly preferred that the nucleotide molecule comprises the sequence TGACTCA.

The present invention is now illustrated in the appended examples with reference to the following figures.

Figure 1

DNA sequence of Fos regulated gene F0401, showing the encoded protein sequence and the regions homologous to VEGF (underlined).

Figure 2

DNA sequence of Fos regulated gene HF175 (human homologue of F0401), showing the encoded protein.

Figure 3

Alignment of the protein encoded by FIGF with the conserved domain of the PDGF/VEGF family of growth factors. Dots indicate the cysteine residues which are characteristic of these growth factors.

Figure 4

(A) Immunoprecipitation assay of the FIGF protein. COS-7 cells transfected with the vector alone (-) or with a vector containing the FIGF coding sequence under the control of a CMV promoter (+) were metabolically labelled for 1 hour with [^{35}S]Methionine and [^{35}S]Cysteine each at a concentration of 100 μCi/ml. After 1 hour or 22 hours chase, conditioned media and cell lysates were immunoprecipitated separately with anti-FIGF polyclonal antibodies. [The FIGF protein was expressed in E. coli under the control of the T5 promoter. The cDNA fragment, from the coding region of FIGF,

was generated by PCR from the Methionine residue at position +40 and cloned into the pQE-31 vector (Qiagen) to obtain a fusion protein with a N-terminal Histidine tag. The protein was expressed in TG1 bacteria (pREP+) by induction for 4 hours at 37 °C in the presence of 2mM isopropyl-b-D-thiogalactopyranoside. The recombinant protein was exclusively localized in inclusion bodies, and was purified on a column of Ni-NTA-Resin, under denaturing conditions according to the manufacturer's protocols (Qiagen). Antibodies were raised by injecting rabbits with 200 μg of recombinant FIGF in form of denaturated protein in complete Freund's adjuvant. Serum was prepared after 4 injections in incomplete Freund's adjuvant at 3-week intervals]. The immunocomplexes were collected by protein-A Sepharose beads (Pharmacia) and separated on 12% SDS-PAGE in the presence of 3% b-mercaptoethanol. Arrows indicate specific bands present only in FIGF transfected cells.

(B) Mitogenic activity measured as [3H]-thymidine incorporation in c-fos (-/-) fibroblasts. Cells were incubated with conditioned medium of COS-7 cells transfected with the FIGF expression vector or with the vector alone. One day after transfection the cells were split and kept in 2% serum. Conditioned media were collected after 120 hours.

(C) Mitogenic activity measured as [3H]-thymidine incorporation in c-fos (-/-) fibroblasts. Cells were incubated with conditioned media obtained from c-fos (-/-) stable clones, named FH-10.2, FH-10.5, FH-9.3, FH-9.6, FH-10.9 and c-fos (-/-) cells (mock), constitutively expressing exogenous FIGF under the control of the CMV promoter. Conditioned media were collected from cells cultured for 48 hours in 0.5% serum.

(D) Mitogenic activity measured as [3H]-thymidine incorporation in c-fos (-/-) fibroblasts. Cells were incubated with partially renatured recombinant FIGF. Under the same conditions, incubation with PDGF-BB (Sigma), used as a positive control, induces about 30 % higher thymidine incorporation, while VEGF (Sigma) does not induce incorporation above the background. The data shown are the

mean of six experiments performed with two different FIGF preparations.

(E) Mitogenic activity measured as [3H]-thymidine incorporation on mouse embryo fibroblasts. Cells were incubated with partially renatured recombinant FIGF. MEF cells were obtained from 13-15 day embryos of B6D2F1 mice. The embryos were sacrified, rinsed and trypsinized for 30 min at 37°C. The MEF cells were grown 24 hours in medium containing 0.5 % serum before addition of the growth factors. Under the same conditions, incubation with PDGF-BB (Sigma), used as a positive control, induces about 30 % higher thymidine incorporation, while VEGF (Sigma) does not induce incorporation above the background. The data shown are the mean of six experiments performed with two different FIGF preparations. The background values were subtracted in each experiment.

Figure 5

(A) Expression of FIGF in cultured cells. Northern blot analysis of total RNA obtained from: c-fos (-/-) fibroblasts (lanes 1 - 3); a stable cell line, obtained from c-fos (-/-) cells, expressing exogenous c-fos (lanes 4 - 6), c-fos (+/+) fibroblasts (lanes 7 - 9). Cellular RNA was extracted by the guanidine thiocyanate method after incubation of cells for 48 hours in 0.5 % serum (time 0). The serum concentration was increased to 10 % and total RNA was collected at 2 or 4 hours as indicated. Lanes 10 and 11 show FIGF expression in c-fos (-/-) fibroblasts transiently transfected with the vector alone (mock) or containing the c-fos under the FBJ-LTR constitutive promoter (c-fos). The RNAs of the transiently transfected cells were collected 30 hours after culturing the cells in medium containing 0.5% serum. Each lane was loaded with 10μg of total cellular RNA.

(B) Expression of PDGF or VEGF. Total cellular RNAs from c-fos (-/-) cells (lanes 1-3) or from a stable cell line, obtained from c-fos (-/-) cells, expressing exogenous c-fos (lanes 4 - 6) were extracted as indicated in panel A.

Glyceraldehyde-phosphate-dehydrogenase (GAPDH) was used as a control for RNA loading.

Figure 6

Northern analysis of RNA poly A+ extracted from different mouse tissues.

Figure 7

(A) Morphology of c-fos deficient cells. The cells were stably transfected with the vector alone.

(B) Morphology of a cell clone derived from c-fos deficient cells, stably transfected with the expression vector containing FIGF under the control of the CMV promoter.

(C) Morphology of cells stably transfected with an expression vector containing the FIGF cDNA in the antisense orientation under the control of the CMV promoter.

(D) Morphology of cells stably transfected with the expression vector containing c-fos under the control of the FBJ-LTR promoter.

(E) A cell clone derived from the same cells as in D (expressing c-fos contitutively) transfected with an expression vector containing FIGF under the control of the CMV promoter.

(F) A cell clone derived from the same cells as in D (expressing c-fos contitutively) transfected with an expression vector containing the FIGF cDNA in the antisense orientation under the control of the CMV promoter.

(G) c-fos (-/-) fibroblasts cultured for 120 hours in medium containing 0.5 % serum.

(H) Cells as in G but treated for 120 hours with partially renatured recombinant FIGF. Ten independent clones obtained from 3 independent transfections were analyzed. All showed morphological changes similar to those observed in the figure.

EXAMPLES

Cell Culture and Clone Isolations

Mouse fibroblast wild-type cells with respect to c-Fos

expression (+/+) and c-Fos-deficient (-/-) 3T3 cell lines and stably transfected cell line that constitutively express exogenous c-Fos were generated as described (Hu, *et al.*, 1994). All cell lines were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine and penicillin-streptomycin. Cells were cultured until reaching about 70% confluence, serum starved for 48 hours in DMEM containing 0.5% FCS and stimulated with DMEM containing 10% FCS for 0, 2 and 4 hours prior to RNA isolation. Total RNA was isolated using the quanidine-isothiocyanate method. mRNA differential display was performed as described by Laing *et al.* and modified by Bauer *et al.* (Bauer *et al.*, 1993). Briefly, from the extracted RNA chromosomal DNA contamination was removed from 50 μg of the total RNA isolated by DNase I treatment. 0.2 μg of RNA, extracted at 2 or 4 hours after serum induction, was used for reverse transcription in a 40μl reaction volume using $dT_{12}mN$ primers and 300 U MMLV reverse transcriptase (Promega Corp., Madison, WI) with an incubation time of 60 min at 37°C. The PCR mixture for the cDNA amplification contained $dT_{12}mN$ primer, one of the 20 10mer deoxyoligonucleotide primers with arbitrary sequence (Kit A - Operon Biotechnology Inc., Alameda, CA), ^{33}P-dATP (Amersham International plc, Buckinghamshire, England) and 1U Taq polymerase (Promega Corp.). Samples were subjected to 40 cycles of amplification using a PTC-100 thermocycler (MJ Research Inc., Watertown, MA). The cycling parameters were as follows: 94°C for 30 seconds, 42°C for 90 seconds, 72°C for 30 seconds and an additional extension period at 72°C for 10 min. 2 μl of the PCR mixture was adjusted with glycerol to 5% and loaded onto a 6% polyacrylamide gel without urea (Bauer *et al.*, 1993). The bands of cDNA differentially expressed, were recovered from the gel and reamplified. Reamplified cDNA probes were run on a 1.5% agarose gel, purified and cloned into the pGEM-T vector using the TA cloning system (Promega Corp.) Positive clones were selected using the blue-white phenotype.

Characterisation and Sequencing of Novel Clones

Typically from one band we could obtain 1 to 3 different clones, which we utilized for the successive characterisation by Northern blot analysis. The cDNA fragments were labelled with ^{32}P-dCTP using a random primer labelling kit (Amersham International plc). Hybridisation signals were screened and quantitated by PhosphorImager using Image Quant software (Molecular Dynamics, Sunnyvale, CA). Plasmid DNA sequencing of cloned cDNA probes with either T7 or SP6 primer was carried out manually using the Sequenase V 2.0 Kit (US Biochemical Inc., Cleveland, Ohio). Briefly, the RNA extracted from the cells were subjected to amplification utilising random primers and the bands of a cell type are identified by comparison and isolated. The fragments obtained were tested in Northern blot with RNA from the cell lines to confirm that the corresponding mRNA are up regulated in Fos expressing cells. Then we generated our own cDNA library in lambda ZAP vectors from mouse fibroblasts cell lines to obtain the full length clones utilising a cDNA Synthesis and Cloning Kit (Stratagene). The screening was performed according to the manufacturer. Positive clones were first analysed by restriction map and the longest ones were subjected to DNA sequence.

Clone Analysis

F0401 sequence is shown in Figure 1 and the HF175 sequence is shown in Figure 2: a simple search analysis against the NIH and EMBL data banks revealed that F0401 and the human homologue FIGF are novel genes and their sequences are similar to the genes of a family of growth factors characterised by the Platelet growth Factor (PDGF) family signature. The consensus pattern of the family is: C-V-x(3)-R-C-x-G-C-C-N.

Members of this family form dimers with disulphide links and are potent mitogens. The most similar sequence to F0401 and HF175 is the Vascular Endothelial growth factor (VEGF) which forms an homodimer and is a growth factor active in

angiogenesis and endothelial cell growth (Keck *et al.*, 1989; Leung *et al.*, 1989). As VEGF is a growth factor its over expression can result in tumour angiogenesis (Plate *et al.*, 1993). Recent reports indicate possible therapeutic use based on VEGF inhibition in tumours (Kim *et al.*, 1993) and on VEGF treatment to stimulate angiogenesis (Takeshita *et al.*, 1994).

The following experiments were performed using F0401.

The FIGF predicted protein sequence has a hydrophobic sequence at the N-terminus which could code for a signal peptide. This long N-terminius region does not show significant homology to known proteins. However, there is a positively charged domain in this region which may allow binding of the protein to the cell membrane or to the extracellular matrix.

To verify if FIGF is a secreted protein, we transfected COS-7 cells with an expression vector containing the FIGF cDNA under the control of the cytomegalovirus (CMV) immediate early gene promoter. Polyclonal antibodies, raised against recombinant FIGF (as described previously), immunoprecipitated a specific band that is observed in both the cell lysates and the conditioned media of the FIGF transfected COS-7 cells (Fig.4A). After 1 hour labelling followed by 1 hour chase a specific band was mainly present in the cell lysate while, after a chase longer than four hours, the protein accumulated in the cell supernatant. Under non-denaturing conditions FIGF aggregated into a multimeric form. Addition of b-mercaptoethanol resulted in partial denaturation of the protein which migrated mostly as a 66 kDa band and only a minor fraction of the protein can be found as a monomer of the expected 33 kDa of molecular mass (Fig.4A). These results show that FIGF is a secreted protein and can form dimers. Dimerization of FIGF could be predicted since the FIGF central domain is highly conserved and contains the cysteine residues involved in the

dimerization of both PDGF and VEGF. It was further investigated whether the conditioned medium of FIGF producing cells could promote cell growth in vitro, assayed as [3H]-thymidine incorporation (Vaziri *et al.* (1995)). Conditioned medium was obtained either from transiently transfected COS-7 cells or from stable clones, derived from c-fos(-/-) fibroblasts, expressing FIGF under the control of the CMV promoter. The mitogenic activity of the medium containing FIGF was tested on c-fos(-/-) fibroblasts. Conditioned medium from both transfected COS-7 (Fig. 4B) or stable fibroblast clones overexpressing FIGF (Fig. 4C) induces DNA synthesis in c-fos (-/-) fibroblasts. As in mammalian cells FIGF expression could induce the activation of other growth factors, which in turn would be responsible for the [3H]-thymidine incorporation measured, we tested the mitogenic activity of a recombinant FIGF protein expressed in E. coli (as described previously). In order to obtain a biologically active recombinant protein, the purified FIGF protein from E. coli was partially renatured in the presence of a mixture of reduced and oxidized glutathione. The purified recombinant protein was adjusted to 0.4 mg/ml and completely reduced in the presence of 8M Urea, 2% b-mercaptoethanol for 1 hour at 370 °C. The reduced protein was dialized against a solution containing 50 mM Tris-Cl pH 8.0, 1M Urea, 5 mM reduced glutathione and 0.5 mM oxidized glutathione for 2 days, and against a solution containing 20 mM Tris-Cl pH 7.5, 0.7 M NaCl for 1 day, as described by Hoppe *et al.*, Biochemistry, 28, pp 2956-2960 (1989); Hoppe *et al.*, Eur. J. Biochem., 187, pp 207-214 (1990). The partially refolded recombinant FIGF induced DNA synthesis on c-fos (-/-) fibroblasts in a dose-dependent manner (Fig. 4D). As expected, c-fos(-/-) cells are also responsive to PDGF-BB, while the treatment with VEGF did not induce [3H]-thymidine incorporation in these cells. The highest activity of DNA synthesis was obtained with 2 μg of purified FIGF. The apparently low specific activity of the recombinant FIGF observed, is most probably due to the low efficiency of FIGF correct refolding since FIGF contains 29

cysteine residues out of 358 amino acids. We also tested the mitogenic activity of the recombinant FIGF on mouse embryo fibroblasts (MEF). FIGF induced DNA synthesis on mouse embryo fibroblasts in a dose-dependent manner (Fig. 4E) . The FIGF cDNA was isolated by differential screening of RNA from cells differing only for the expression of c-fos. Analysis of FIGF gene expression by Northern blot reveals that the FIGF messenger is barely detectable in c-fos (-/-) fibroblasts, while its expression is high in wild type c-fos (+/+) fibroblasts (Fig. 5A, compare lanes 1-3 with lanes 7-9). FIGF expression is completely restored in stable clones, derived from c-fos (-/-) cells, expressing exogenous c-fos under the control of the FBJ-LTR constitutive promoter (Hu *et al.* (1994)) (Fig. 5A, compare lanes 1-3 with lanes 4-6). The transient transfection of exogenous c-fos results in FIGF induction in c-fos (-/-) cells, although, due to the lower number of transfected cells, the induction observed is less pronounced (Fig . 5A lanes 10 and 11). Thus, FIGF expression is dependent on c-fos. Moreover, FIGF is not induced by the constitutive AP-1 yeast homologue GCN4. In mammalian cells GCN4 is able to activate most AP-1 target genes, but it is non-oncogenic. In wild type fibroblasts c-Fos is the major Fos protein associated with c-Jun or Jun B within the first hour after serum induction. Afterwards c-Fos is not detectable any longer and it is substituted by FraJ1 and FraJ2 in the AP-1 complex. In c-fos expressing cells, FIGF is highly expressed when cells are kept in low serum conditions and decreases to undetectable levels within six hours after serum induction (Fig. 5A). This pattern of FIGF expression can be observed both in wild type cells and in cells constitutively expressing c-fos (Fig. 5A). Thus, we observe a discrepancy between the expected peak of c-fos expression and the appearance of FIGF, whose messenger accumulates in the quiescent phase. The FIGF pattern of regulation suggests that, besides the expression of c-fos, additional regulatory controls are required for its activation. Although FIGF belongs to the PDGF/VEGF family of growth factors, its

expression is most similar to the expression of the growth arrest specific (gas) genes. Interestingly, one of them, gas6, acts as a growth factor. Both PDGF and VEGF growth factors are involved in tumour formation (Kim *et al.* (1993)). Moreover, PDGF is the main serum mitogen which induces the transcription activation of c-fos. In order to compare the pattern of expression of these growth factor with respect to FIGF, we measured the PDGF and VEGF messengers levels in fibroblasts differing for the expression of c-fos. As can be observed in Fig. 5B, the regulation of both PDGF and VEGF messengers is distinct from that of FIGF. These growth factors are rapidly induced following serum induction and their expression is independent of c-fos. Tumour progression is characterized by morphological changes of the tumour that leads the mutated cells to loose their adhesion to the original neighbours and escape from the tissue of origin. c-fos has been implicated in tumour progression and its over-expression induces a transformed cell morphology in fibroblasts and epithelial cells. As FIGF is a c-fos-dependent growth factor, it was analyzed whether its over-expression could induce fibroblast morphological transformation. As can be observed in Fig. 7, the constitutive expression of FIGF in fibroblasts induces a transformed phenotype. Stable clones derived from c-fos (-/-) cells, costitutively expressing FIGF, acquire a spindle-shaped morphology, become more refractive, and detach from the plate (Fig 7, B versus A). On the contrary, stable clones expressing the FIGF antisense messenger acquire a flat and less rifrangent phenotype (Fig. 7C), which is most similar to the phenotype of c-fos (-/-) cells kept in low serum conditions (Fig.7G). The over-expression of c-fos alters c-fos (-/-) cell morphology similarly to that observed with the over-expression of FIGF, although the phenotye is less pronunced (Fig. 7D). The over-expression of both c-fos and FIGF leads to an extreme phenotype in fibroblasts: cells become longer, disorganized and lose contacts (Fig.7E). The expression of the FIGF

antisense messenger in cells constitutively expressing c-fos induces a reversion of the transformed phenotype (Fig. 7F). Thus, cells expressing c-fos but depleted of FIGF loose most of the transformed phenotype, suggesting that the morphology observed in cells constitutively expressing c-fos is due to FIGF. Similar morphological alterations are also obtained by cell treatment with purified recombinant FIGF. c-fos (-/-) fibroblasts, kept in medium containing 0.5% serum for 120 hours stop growing, become flat large and less rifrangent (Fig.7G). Cell treatment with recombinant FIGF induces the rifrangent, elongated and non-adherent phenotype (Fig.7H).

Tumours obtained from cells defective for c-fos cannot undergo malignant progression even if they are carrying the activated v-H-Ras. Thus, the expression of c-fos is essential for the activation of target genes responsible for the malignant phenotype. FIGF is a c-fos-dependent autocrine growth factor able to induce cell division entry and, when it is over-expressed, a transformed phenotype in fibroblasts. The data suggest that the role of c-fos in the activation of the malignant phenotype is due to the activation of FIGF.

Further experiments on FIGF using a probe specific for FIGF in Northern analysis of RNA derived from mouse tissues, show that the FIGF gene is only expressed in cells expressing Fos and poorly in cells that lack the Fos oncogene (Figure 5). The RNA blot used in the Northern assay was obtained from Clontec. The analysis of its expression in the mouse tissues shows that FIGF is mainly expressed in lung (Figure 6) and is already present at day 7 of the mouse embryonal life (not shown).

FIGF is therefore a molecule related to the growth factor VEGF, positively regulated by the oncogene Fos. It could be implicated in tumours and in development.

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43. EP-A-0125023

Claims

1. A nucleotide molecule encoding a protein encoded by a Fos regulated gene or a fragment thereof, wherein said protein or fragment thereof is encoded by any one of the nucleotide sequences shown in Figure 1 or 2 or a fragment thereof, including allelic variants and species variants of the nucleotide sequences.

2. The nucleotide molecule of claim 1, wherein said protein or a fragment thereof, encoded by the sequences shown in Figure 1 or 2 is altered but still has at least 80% homology to the protein or a fragment thereof, encoded by the sequences shown in Figure 1 or 2.

3. The protein encoded by the nucleotide molecule of claim 1 or claim 2.

4. A vector for the expression of the nucleotide molecule of claim 1 or claim 2, comprising a promoter and said nucleotide molecule.

5. A host cell transformed with the vector of claim 4.

6. The host cell of claim 5 which is a chinese hamster ovary cell.

7. A method for the producing the protein of claim 3, comprising culturing the host cell of claim 5 or claim 6 under conditions leading to the production of the protein and harvesting the protein.

8. The nucleotide molecule of claim 1 or claim 2 for use in therapy.

9. The use of the nucleotide molecule of claim 1 or claim 2 in the manufacture of a composition for the treatment of

developmental disorders.

10. An antibody molecule having specificity for the protein of claim 3.

11. The antibody molecule of claim 10 for use in therapy.

12. The use of the antibody molecule of claim 10 in the manufacture of a composition for the treatment of proliferative diseases.

13. An antisense nucleotide molecule having the complementary sequence to the nucleotide molecule of claim 1 or claim 2.

14. An antisense vector for the expression of the antisense nucleotide molecule of claim 13, comprising a promoter and the antisense molecule.

15. The antisense vector of claim 14 for use in therapy.

16. The use of the antisense vector of claim 14 in the manufacture of a composition for the treatment of proliferative diseases.

17. A vector for the expression of a ribozyme, comprising a promoter and a nucleotide sequence encoding a ribozyme capable of cleaving the RNA transcript of the nucleotide molecule of claim 1 or claim 2.

18. The vector of claim 17 for use in therapy.

19. The use of the vector of claim 17 in the manufacture of a composition for the treatment of proliferative diseases.

20. The use of claim 12, 16 or 19, wherein the proliferative disease is cancer.

21. The use of the protein of claim 3 in identifying the receptor of said protein.

22. The use of the protein of claim 3 in an assay for the identification of antagonists or agonists of said protein.

23. The use of the nucleotide molecule of claim or claim 2, the protein of claim 3 or the antibody molecule of claim 10 in diagnosing a pathological state or a predisposition to a disease.

24. The use of the nucleotide sequence of claim 1 or claim 2 in the generation of a transgenic animal.

25. A nucleotide molecule comprising all or part of the sequence shown in any one of Figures 1 or 2.

FIG. 1

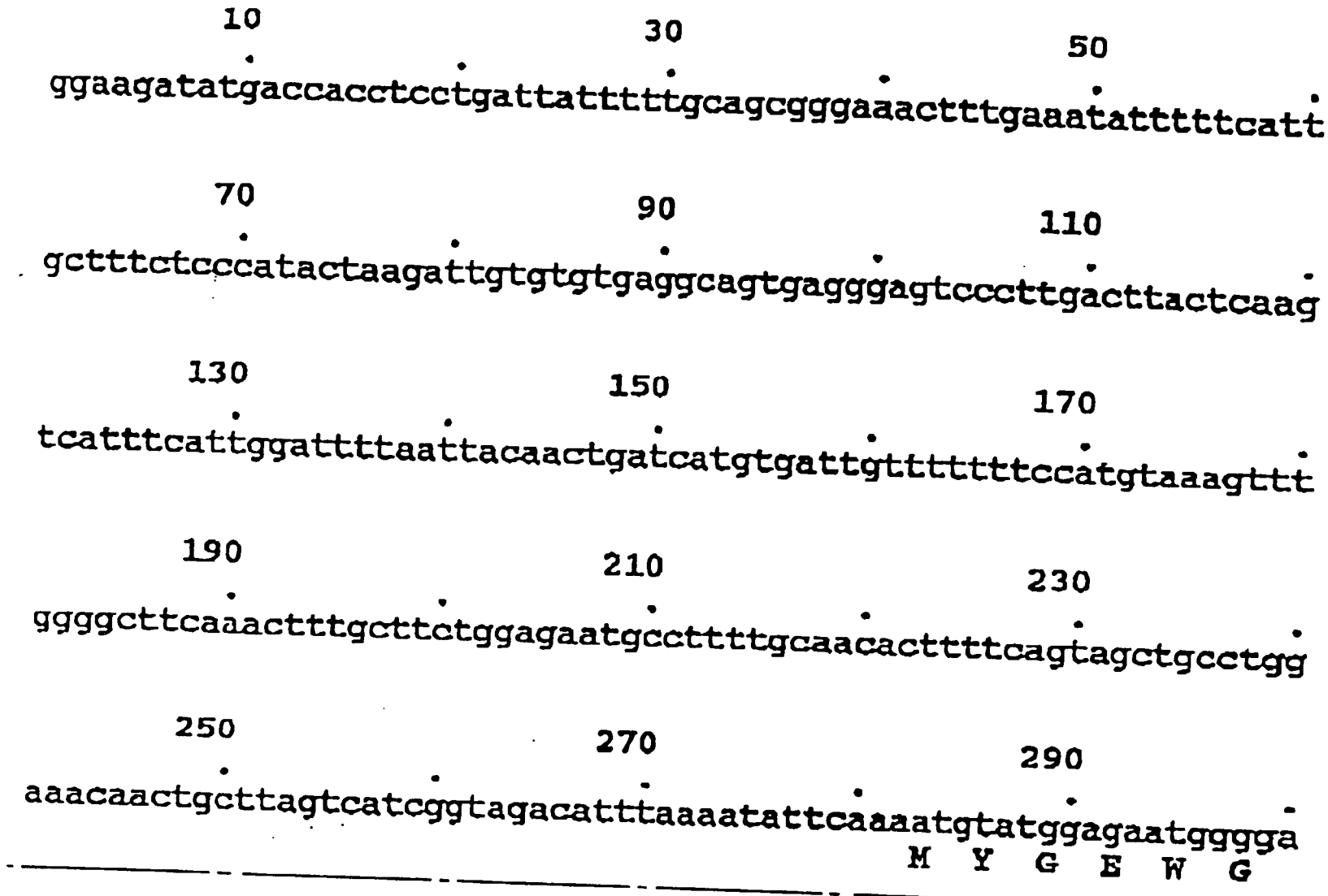

10
30
50
ggaagatatgaccacctcctgattatttttgcagcgggaaactttgaaatatttttcatt
70
90
110
gctttctcccatactaagattgtgtgtgaggcagtgagggagtcccttgacttactcaag
130
150
170
tcatttcattggattttaattacaactgatcatgtgattgtttttttccatgtaaagttt
190
210
230
ggggcttcaaactttgcttctggagaatgcctttttgcaacacttttcagtagctgcctgg
250
270
290
aaacaactgcttagtcatcggtagacatttaaaatattcaaaatgtatggagaatgggga
M Y G E W G

FIG. 1(i)

```
310                330                350
atggggaatatcctcatgatgttccatgtgtacttggtgcagggcttcaggagcgaacat
M  G  N  I  L  M  M  F  H  V  Y  L  V  Q  G  F  R  S  E  E  H

370                390                410
ggaccagtgaaggatttttcttttgagcgatcatcccggtccatgttggaacgatctgaa
G  P  V  K  D  F  S  F  E  R  R  S  S  R  S  M  L  E  R  S  E

430                450                470
caacagatccgagcagcttctagtttggaggagttgctgcaaatcgcgcactctgaggac
Q  Q  I  R  A  A  S  S  L  E  E  L  L  Q  I  A  H  S  E  D
```

FIG. 1(II)

```
      490                 510                 530
       .         .         .         .         .         .
tggaagctgtggcgatgccggttgaagctcaaaagtcttgccagtatggactcacgctca
W  K  L  W  R  C  R  L  K  L  K  S  L  A  S  M  D  S  R  S

      550                 570                 590
       .         .         .         .         .         .
gcatcccatcgctccaccagatttgcggcaactttctatgacactgaaacactaaaagtt
A  S  H  R  S  T  R  F  A  A  T  F  Y  D  T  E  T  L  K  V

      610                 630                 650
       .         .         .         .         .         .
atagatgaagaatggcagaggacccaatgcagccctagagagacatgcgtagaagtcgcc
I  D  E  E  W  Q  R  T  Q  C  S  P  R  E  T  C  V  E  V  A

      670                 690                 710
       .         .         .         .         .         .
agtgagctggggaagacaaccaacacattcttcaagccccctgtgtaaatgtcttccgg
S  E  L  G  K  T  T  N  T  F  F  K  P  P  C  V  N  V  F  R
```

FIG. 1(III)

```
        730                 750                 770
        .                   .                   .
tgtggaggctgctgcaacgaagagggtgtgatgtgtatgaacacaagcacctcctacatc
C  G  G  C  C  N  E  E  G  V  M  C  M  N  T  S  T  S  Y  I

        790                 810                 830
        .                   .                   .
tccaaacagctctttgagatatcagtgcctctgacatcagtgcccgagttagtgcctgtt
S  K  Q  L  F  E  I  S  V  P  L  T  S  V  P  E  L  V  P  V

        850                 870                 890
        .                   .                   .
aaaattgccaaccatacgggttgtaagtgcttgcccacgggcccccgccatccttactca
K  I  A  N  H  T  G  C  K  C  L  P  T  G  P  R  H  P  Y  S

        910                 930                 950
        .                   .                   .
attatcagaagatccattcagaccccagaagaagatgaatgtcctcattccaagaaactc
I  I  R  R  S  I  Q  T  P  E  E  D  E  C  P  H  S  K  K  L

        970                 990                 1010
        .                   .                   .
tgtcctattgacatgctgtgggataacaccaaatgtaaatgtgttttgcaagacgagact
C  P  I  D  M  L  W  D  N  T  K  C  K  C  V  L  Q  D  E  T
```

4/31

FIG. 1(IV)

```
      1030                1050                1070
ccactgcctgggacagaagaccactcttacctccaggaacccactctctgtggaccgcac
P  L  P  G  T  E  D  H  S  Y  L  Q  E  P  T  L  C  G  P  H

      1090                1110                1130
atgacgtttgatgaagatcgctgtgagtgcgtctgtaaagcaccatgtccgggagatctc
M  T  F  D  E  D  R  C  E  C  V  C  K  A  P  C  P  G  D  L

      1150                1170                1190
attcagcacccggaaaactgcagttgctttgagtgcaaagaaagtctggagagctgctgc
I  Q  H  P  E  N  C  S  C  F  E  C  K  E  S  L  E  S  C  C

      1210                1230                1250
caaaagcacaagatttttcacccagacacctgcagctgtgaggacagatgtccttttcac
Q  K  H  K  I  F  H  P  D  T  C  S  C  E  D  R  C  P  F  H

      1270                1290                1310
accagaacatgtgcaagtagaaagccagcctgtggaaagcactggcgctttccaaaggag
T  R  T  C  A  S  R  K  P  A  C  G  K  H  W  R  F  P  K  E
```

FIG. 1(v)

```
      1330                1350                1370
acaagggcccagggactctacagccaggagaacccttgattcaacttcctttcaagtccc
T  R  A  Q  G  L  Y  S  Q  E  N  P

      1390                1410                1430
cccatctctgtcattttaaacagctcactgctttgtcaagttgctgtcactgttgcccac

      1450                1470                1490
tacccctgccccccccccctccccgcctccaggtgttagaaaagttgatttgacctagtgt

      1510                1530                1550
catggtaaagccacatttccatgcaatggcggctaggtgattccccagttcactgacaaa

      1570                1590                1610
tgacttgtagcttcagatgtctttgcgccatcagcactcagaaaggaaggggtctgagga
```

```
1630        1650        1670
gccccttgtttgatgaataagaaaaggttgcctgaaacagagtagtaggtgccactcga

1690        1710        1730
ttggttcctcgggctggcaaagtccaagggcaatgctcatgagttattgtgcttctttct

1750        1770        1790
tatgcggaatttcatttgtatgatcagcactgatcaattcccattccacttgtactttttt

1810        1830        1850
aggtttactgaagcactgcctgatgtttttatatgtaaatgtatttaaaggaaataaacac

1870        1890
tgttatgcagcccacaaaaaaaaaaaa
```

FIG.1(VI)

FIG. 2

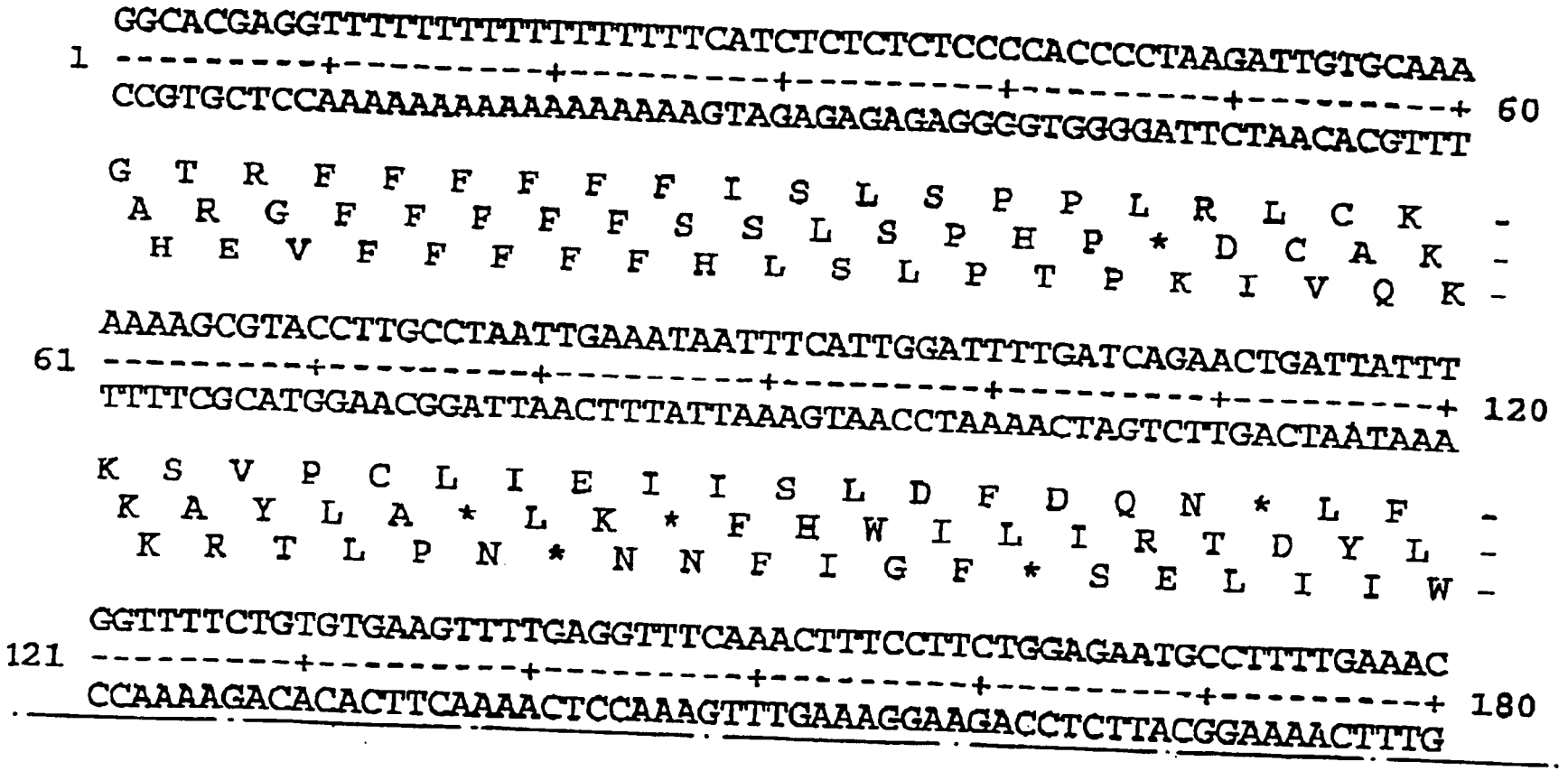


FIG. 2(I)

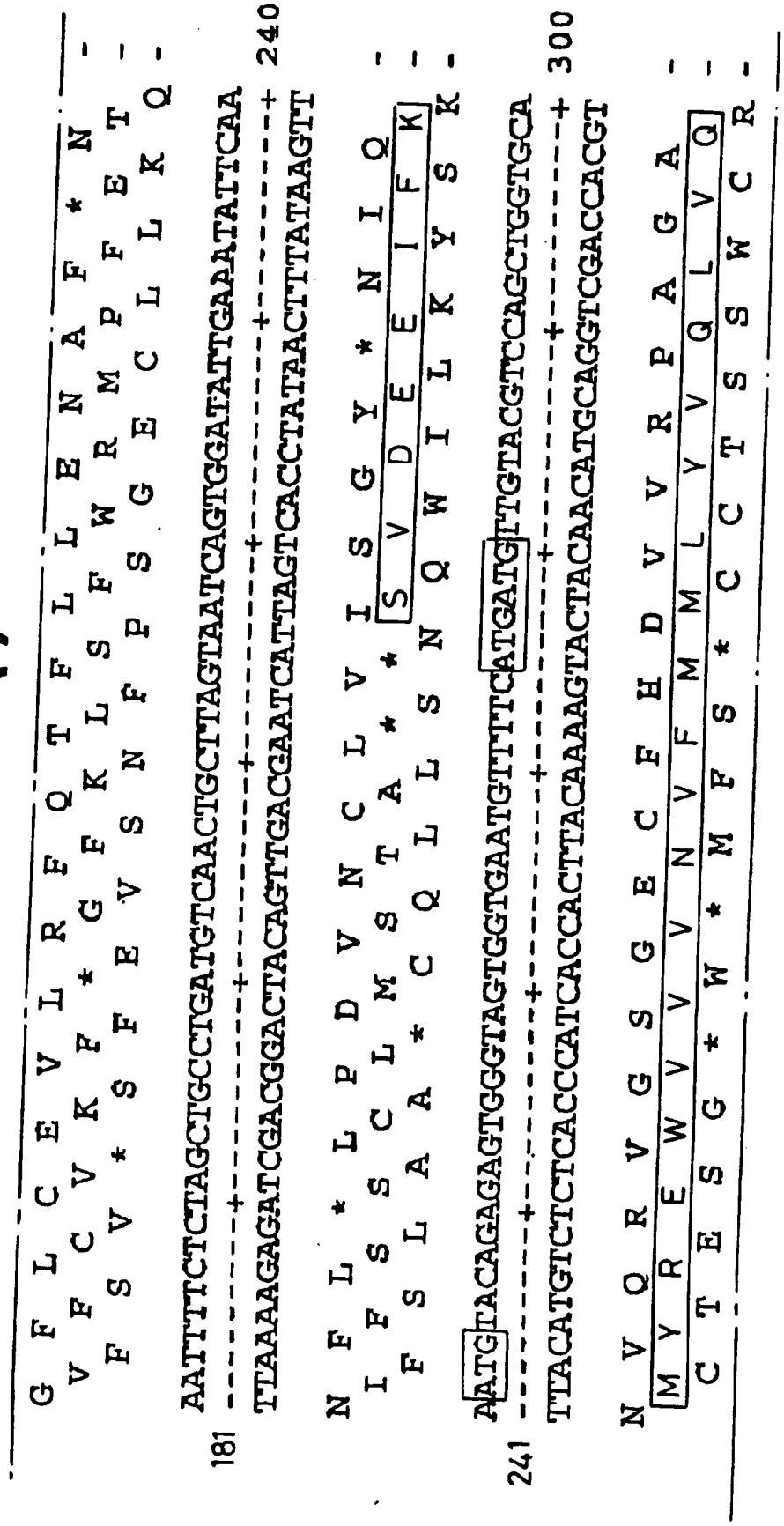

G F L C E V L R F Q T F L L E N A F * N -
V F C V K F * G F K L S F W R M P F E T -
F S V * S F E V S N F P S G E C L L K Q -
AATTTTCTCTAGCTGCCTGATGTCAACTGCTTAGTAATCAGTGGATATTGAAATATTCAA
181 ----------+----------+----------+----------+----------+ 240
TTAAAAGAGATCGACGGACTACAGTTGACGAATCATTAGTCACCTATAACTTTATAAGTT
N F L * L P D V N C L V I S G Y * N I Q -
I F S S C L M S T A * * S V D E E I F K -
F S L A A * C Q L L S N Q W I L K Y S K -
AATGTACAGAGAGTGGGTAGTGGTGAATGTTTTCATGATGTTGTACGTCCAGCTGGTGCA
241 ----------+----------+----------+----------+----------+ 300
TTACATGTCTCTCACCCATCACCACTTACAAAAGTACTACAACATGCAGGTCGACCACGT
N V Q R V G S G E C F H D V V R P A G A -
M Y R E W V V V N V F M M L V V Q L V Q -
C T E S G * W * M F S * C C T S S W C R -

FIG. 2(II)

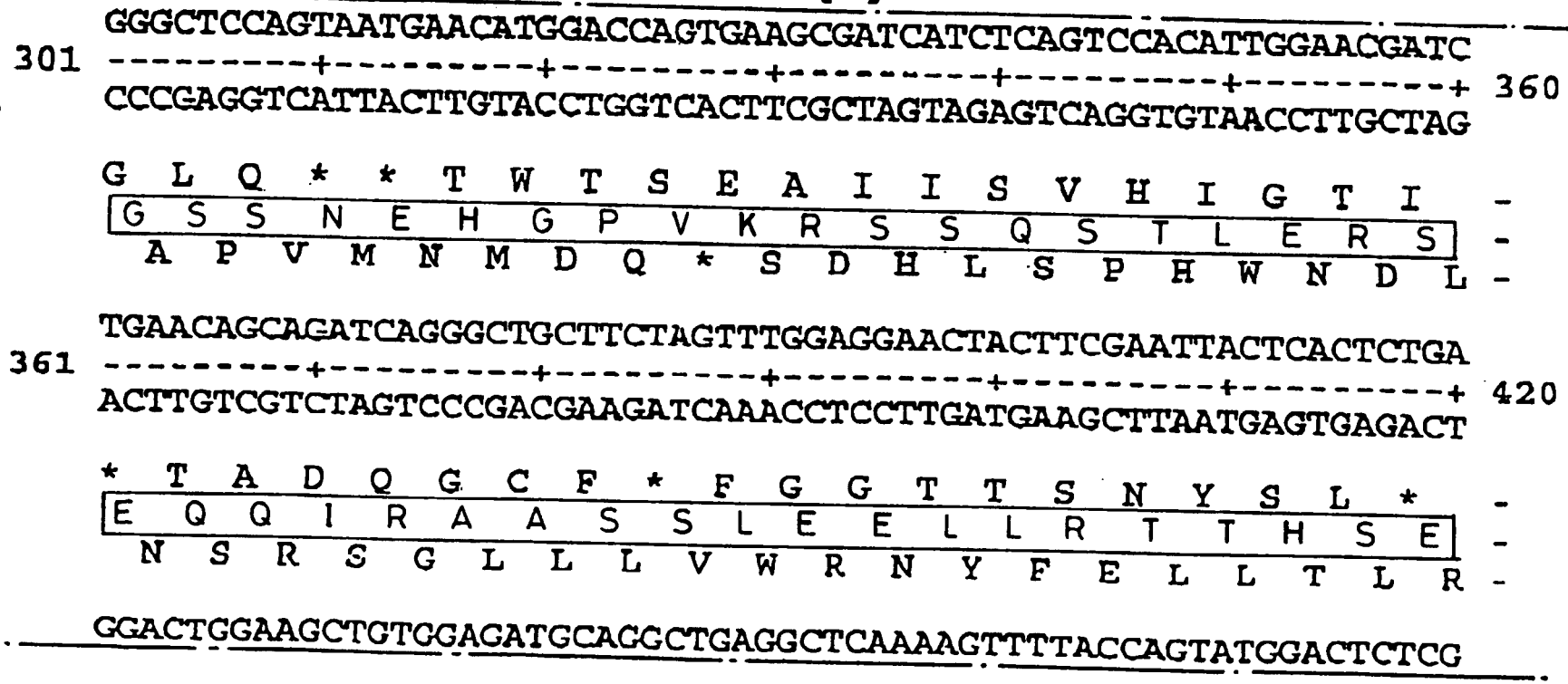

GGGCTCCAGTAATGAACATGGACCAGTGAAGCGATCATCTCAGTCCACATTGGAACGATC
301
360
CCCGAGGTCATTACTTGTACCTGGTCACTTCGCTAGTAGAGTCAGGTGTAACCTTGCTAG
G L Q * * T W T S E A I I S V H I G T I
G S S N E H G P V K R S S Q S T L E R S
A P V M N M D Q * S D H L S P H W N D L
TGAACAGCAGATCAGGGCTGCTTCTAGTTTGGAGGAACTACTTCGAATTACTCACTCTGA
361
420
ACTTGTCGTCTAGTCCCGACGAAGATCAAACCTCCTTGATGAAGCTTAATGAGTGAGACT
* T A D Q G C F * F G G T T S N Y S L *
E Q Q I R A A S S L E E L L R T T H S E
N S R S G L L L V W R N Y F E L L T L R
GGACTGGAAGCTGTGGAGATGCAGGCTGAGGCTCAAAAGTTTTACCAGTATGGACTCTCG

FIG. 2(III)

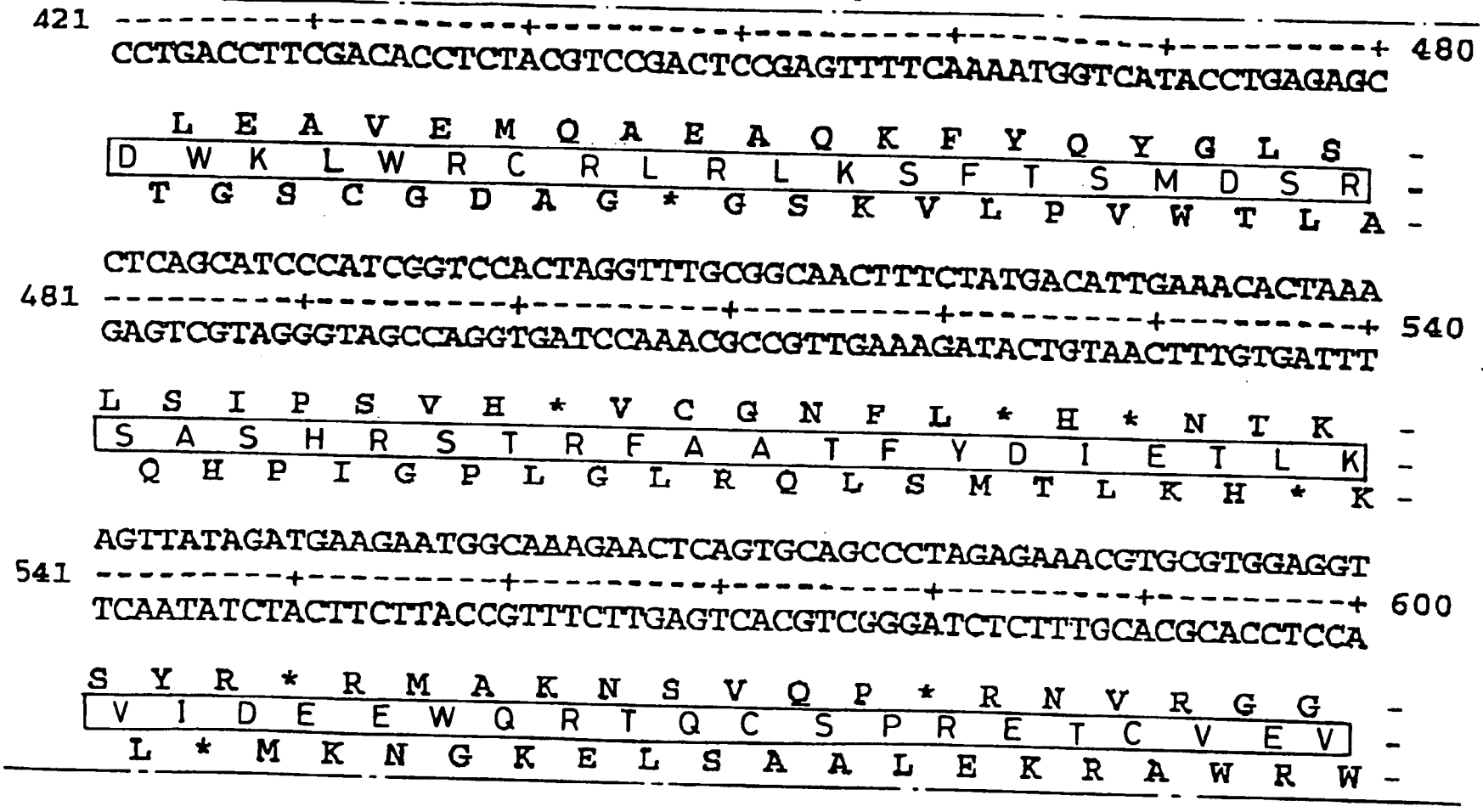

421
480
CCTGACCTTCGACACCTCTACGTCCGACTCCGAGTTTTCAAAATGGTCATACCTGAGAGC
L E A V E M Q A E A Q K F Y Q Y G L S
D W K L W R C R L R L K S F T S M D S R
T G S C G D A G * G S K V L P V W T L A
CTCAGCATCCCATCGGTCCACTAGGTTTGCGGCAACTTTCTATGACATTGAAACACTAAA
481
540
GAGTCGTAGGGTAGCCAGGTGATCCAAACGCCGTTGAAAGATACTGTAACTTTGTGATTT
L S I P S V H * V C G N F L * H * N T K
S A S H R S T R F A A T F Y D I E T L K
Q H P I G P L G L R Q L S M T L K H * K
AGTTATAGATGAAGAATGGCAAAGAACTCAGTGCAGCCCTAGAGAAACGTGCGTGGAGGT
541
600
TCAATATCTACTTCTTACCGTTTCTTGAGTCACGTCGGGATCTCTTTGCACGCACCTCCA
S Y R * R M A K N S V Q P * R N V R G G
V I D E E W Q R T Q C S P R E T C V E V
L * M K N G K E L S A A L E K R A W R W

FIG. 2(IV)

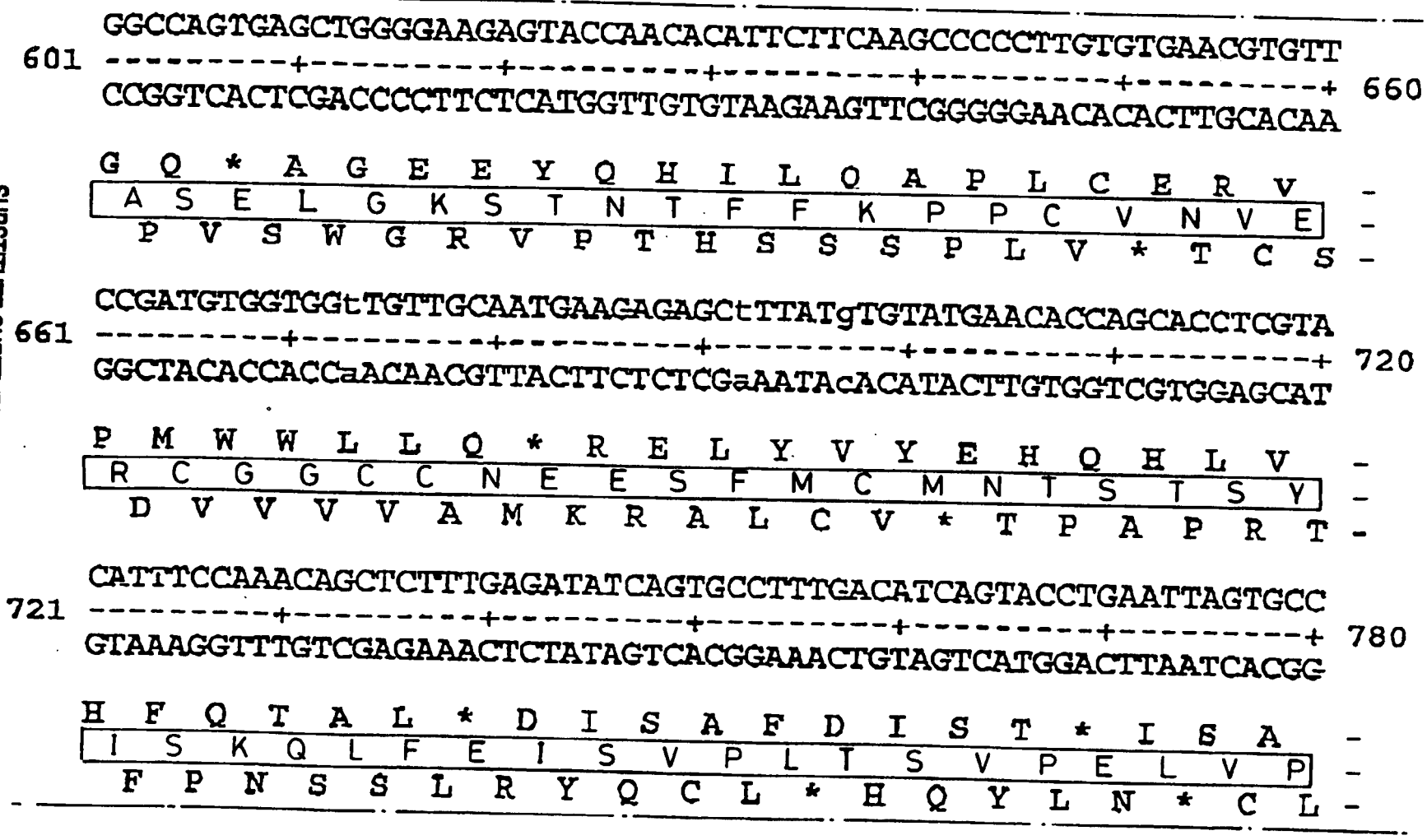

GGCCAGTGAGCTGGGGAAGAGTACCAACACATTCTTCAAGCCCCCTTGTGTGAACGTGTT
601
660
CCGGTCACTCGACCCCTTCTCATGGTTGTGTAAGAAGTTCGGGGGAACACACTTGCACAA
G Q * A G E E Y Q H I L Q A P L C E R V
A S E L G K S T N T F F K P P C V N V E
P V S W G R V P T H S S S P L V * T C S
CCGATGTGGTGGtTGTTGCAATGAAGAGAGCtTTATgTGTATGAACACCAGCACCTCGTA
661
720
GGCTACACCACCaACAACGTTACTTCTCTCGaAATAcACATACTTGTGGTCGTGGAGCAT
P M W W L L Q * R E L Y V Y E H Q H L V
R C G G C C N E E S F M C M N T S T S Y
D V V V V A M K R A L C V * T P A P R T
CATTTCCAAACAGCTCTTTGAGATATCAGTGCCTTTGACATCAGTACCTGAATTAGTGCC
721
780
GTAAAGGTTTGTCGAGAAACTCTATAGTCACGGAAACTGTAGTCATGGACTTAATCACGG
H F Q T A L * D I S A F D I S T * I S A
I S K Q L F E I S V P L T S V P E L V P
F P N S S L R Y Q C L * H Q Y L N * C L

FIG. 2(v)

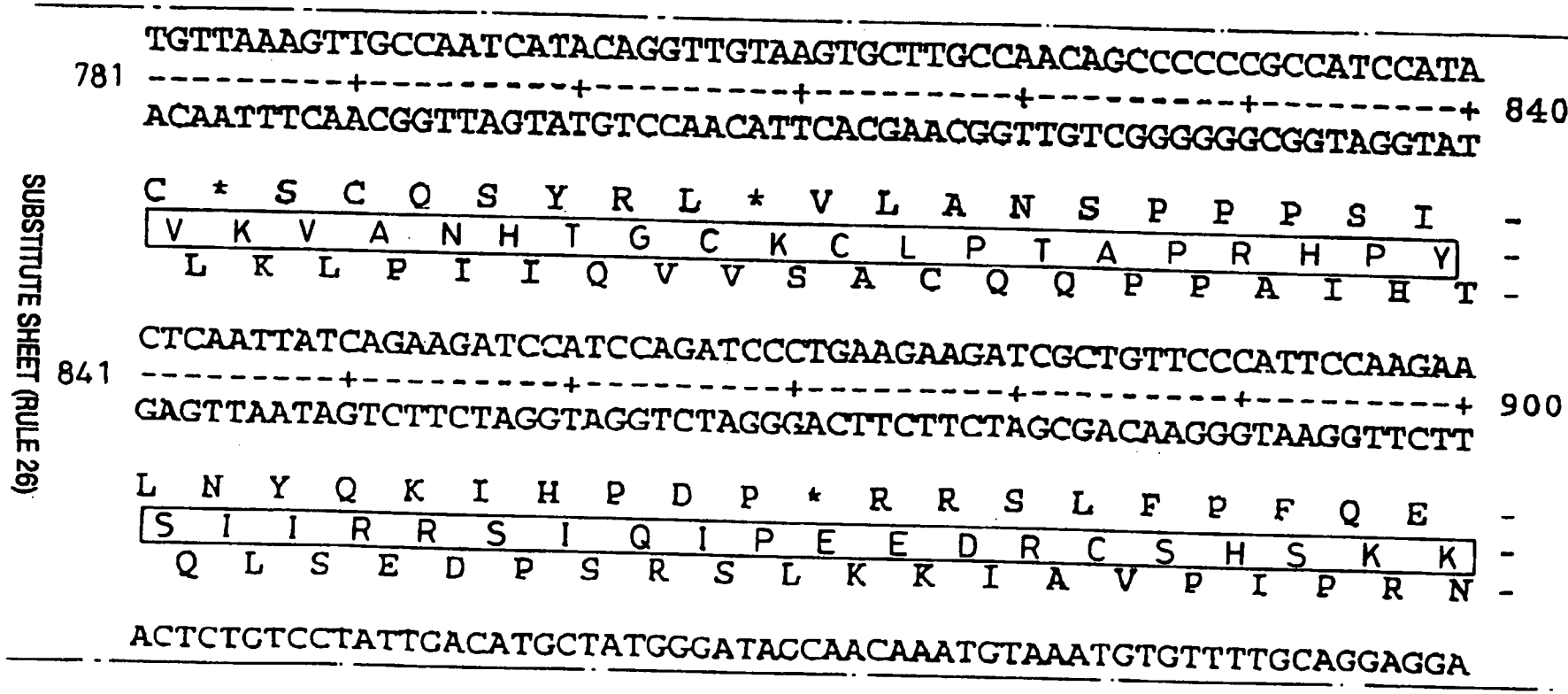


FIG. 2(VI)

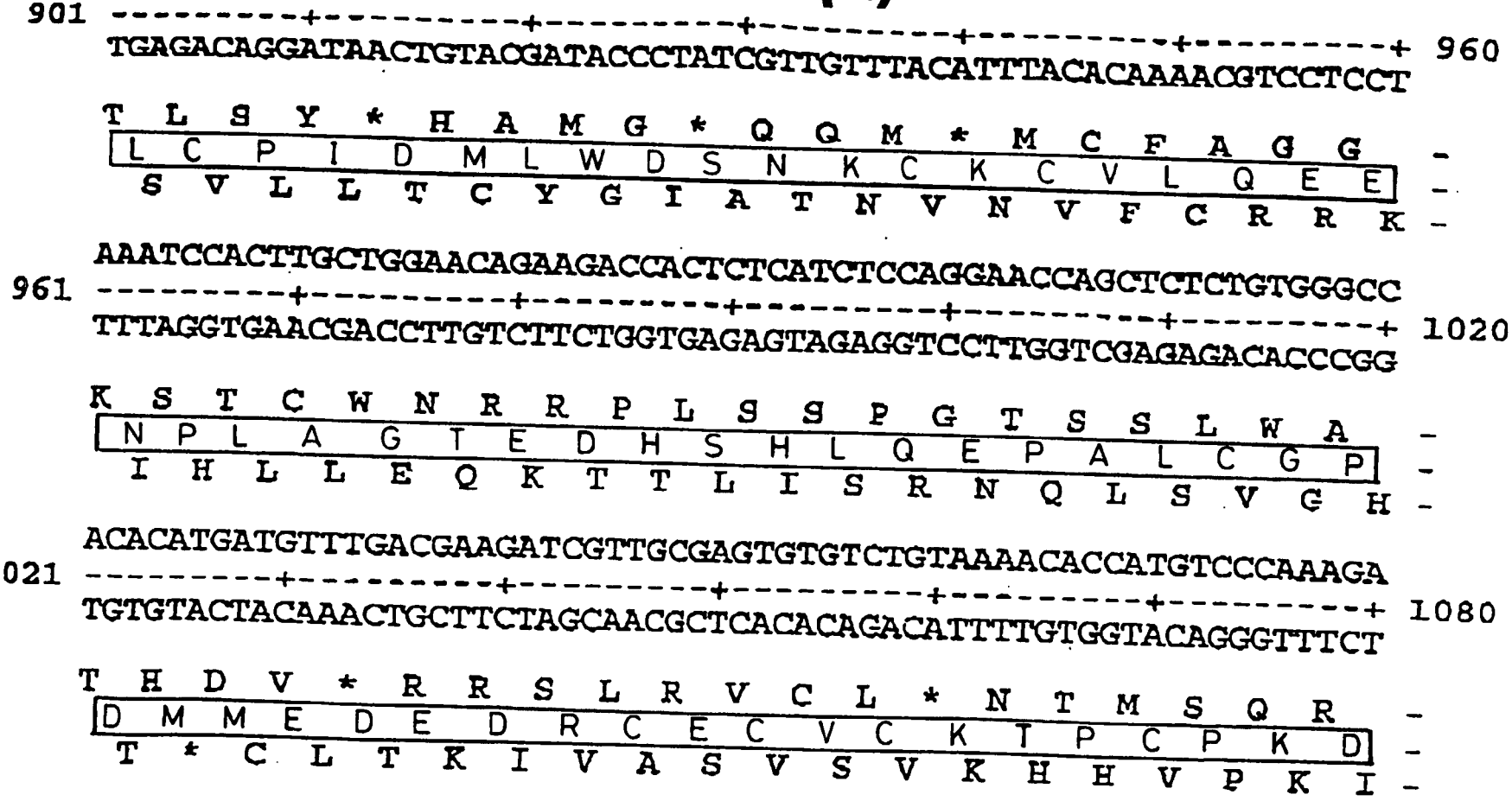

901
TGAGACAGGATAACTGTACGATACCCTATCGTTGTTTACATTTACACAAAACGTCCTCCT
960
T L S Y * H A M G * Q Q M * M C F A G G
L C P I D M L W D S N K C K C V L Q E E
S V L L T C Y G I A T N V N V F C R R K
AAATCCACTTGCTGGAACAGAAGACCACTCTCATCTCCAGGAACCAGCTCTCTGTGGGCC
961
TTTAGGTGAACGACCTTGTCTTCTGGTGAGAGTAGAGGTCCTTGGTCGAGAGACACCCGG
1020
K S T C W N R R P L S S P G T S S L W A
N P L A G T E D H S H L Q E P A L C G P
I H L L E Q K T T L I S R N Q L S V G H
ACACATGATGTTTGACGAAGATCGTTGCGAGTGTGTCTGTAAAACACCATGTCCCAAAGA
1021
TGTGTACTACAAACTGCTTCTAGCAACGCTCACACAGACATTTTGTGGTACAGGGTTTCT
1080
T H D V * R R S L R V C L * N T M S Q R
D M M E D E D R C E C V C K T P C P K D
T * C L T K I V A S V S V K H H V P K I

FIG. 2(VII)

```
                TCTAATCCAGCACCCCAAAAACTGCAGTTGCTTTGAGTGCAAAGAAAGTCTGGAGACCTG
1081            ---------+---------+---------+---------+---------+---------+ 1140
                AGATTAGGTCGTGGGGTTTTTGACGTCAACGAAACTCACGTTTCTTTCAGACCTCTGGAC

                S  N  P  A  P  Q  K  L  Q  L  L  *  V  Q  R  K  S  G  D  L  -
                L  I  Q  H  P  K  N  C  S  C  F  E  C  K  E  S  E  E  T  C  -
                   *  S  S  T  P  K  T  A  V  A  L  S  A  K  K  V  W  R  P  A  -

                CTGCCAGAAGCACAAGCTATTTCACCCAGACACCTGCAGCTGTGAGGACAGATGCCCCTT
1141            ---------+---------+---------+---------+---------+---------+ 1200
                GACGGTCTTCGTGTTCGATAAAGTGGGTCTGTGGACGTCGACACTCCTGTCTACGGGGAA

                L  P  E  A  Q  A  I  S  P  R  H  L  Q  L  *  G  Q  M  P  L  -
                C  Q  K  H  K  L  F  H  P  D  T  C  S  C  E  D  R  C  P  F  -
                   A  R  S  T  S  Y  F  T  Q  T  P  A  A  V  R  T  D  A  P  F  -

                TCATACCAGACCATGTGCAAGTGGCAAAACAGCATGTGCAAAGCATTGCCGCTTTCCAAA
1201            ---------+---------+---------+---------+---------+---------+ 1260
                AGTATGGTCTGGTACACGTTCACCGTTTTGTCGTACACGTTTCGTAACGGCGAAAGGTTT

                S  Y  Q  T  M  C  K  W  Q  N  S  M  C  K  A  L  P  L  S  K  -
                H  T  R  P  C  A  S  G  K  T  A  C  A  K  H  C  R  F  P  K  -
                   I  P  D  H  V  Q  V  A  K  Q  H  V  Q  S  I  A  A  F  Q  R  -
```

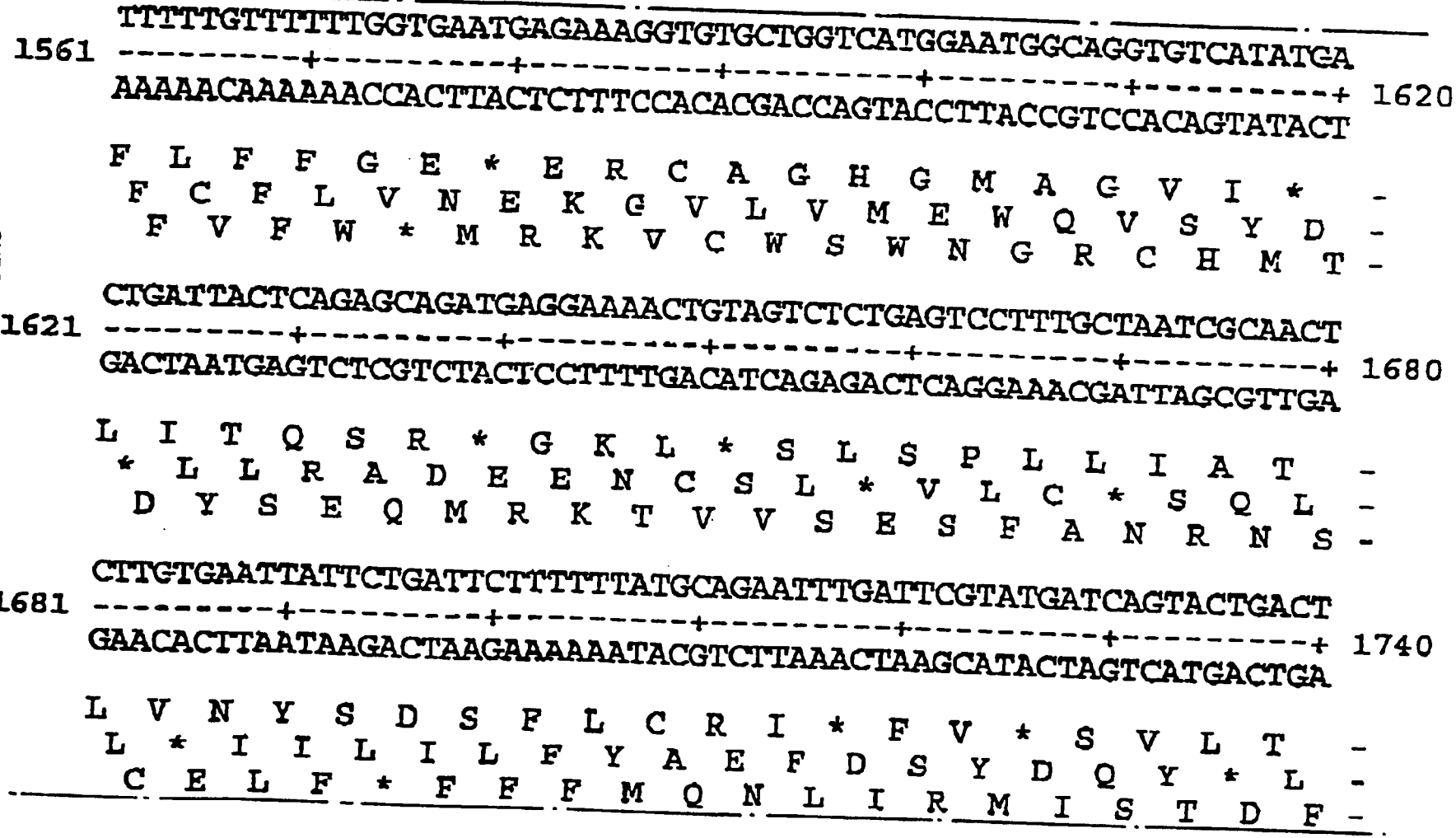
FIG. 2(VIII)

```
                                                                    GGAGAAAAGGGCTGCCCAGGGGCCCCACAGCCGAAAGAATCCTTGATTCAGCGTTCCAAG
1261 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1320
     CCTCTTTTCCCGACGGGTCCCCGGGGGTGTCGGCTTTCTTAGGAACTAAGTCGCAAGGTTC

                                                                    G  E  K  G  C  P  G  A  P  Q  P  K  E  S  L  I  Q  R  S  K  -
                                                                    E  K  R  A  A  Q  G  G  P  H  S  R  K  N  P  *  F  S  V  P  S  -
                                                                    R  K  G  L  P  R  G  P  T  A  E  R  I  L  D  S  A  F  Q  V  -

                                                                    TTCCCCATCCCTGTCATTTTTAACAGCATGCTGCTTTGCCAAGTTGCTGTCACTGTTTTT
1321 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1380
     AAGGGGTAGGGACAGTAAAAATTGTCGTACGACGAAACGGTTCAACGACAGTGACAAAAA

                                                                    F  P  I  P  V  I  F  N  S  M  L  L  C  Q  V  A  V  T  V  F  -
                                                                    S  P  S  L  S  F  L  T  A  C  C  F  A  K  L  L  S  L  F  F  -
                                                                    P  H  P  C  H  F  *  Q  H  A  A  L  P  S  C  C  H  C  F  F  -

                                                                    TTCCCAGGTGTTAAAAAAAAATCCATTTTACACAGCACCACAGTGAATCCAGACCAACC
```

FIG. 2(IX)

```
1381 ---------+---------+---------+---------+---------+---------+
     AAGGGTCCACAATTTTTTTTTAGGTAAAATGTGTCGTGGTGTCACTTAGGTCTGGTTGG

     F  P  G  V  K  K  K  I  H  F  T  Q  H  H  S  E  S  R  P  T  -
      S  Q  V  L  K  K  K  S  I  L  H  S  T  T  V  N  P  D  Q  P  -
       P  R  C  *  K  K  N  P  F  Y  T  A  P  Q  *  I  Q  T  N  L  -

     TTCCATTCACACCAGCTAAGGAGTCCCTGGTTCATTGATGGATGTCTTCTAGCTGCAGAT
1441 ---------+---------+---------+---------+---------+---------+
     AAGGTAAGTGTGGTCGATTCCTCAGGGACCAAGTAACTACCTACAGAAGATCGACGTCTA

     F  H  S  H  Q  L  R  S  P  W  F  I  D  G  C  L  L  A  A  D  -
      S  I  H  T  S  *  G  V  P  G  S  L  M  D  V  F  *  L  Q  M  -
       P  F  T  P  A  K  E  S  L  V  H  *  W  M  S  S  S  C  R  C  -

     GCCTCTGCGCACCAAGGAATGGAGAGGAGGGGACCCATGTAATCCTTTTGTTTAGTTTTG
1501 ---------+---------+---------+---------+---------+---------+
     CGGAGACGCGTGGTTCCTTACCTCTCCTCCCCTGGGTACATTAGGAAAACAAATCAAAAC

     A  S  A  H  Q  G  M  E  R  R  G  P  M  *  S  F  C  L  V  L  -
      P  L  R  T  K  E  W  R  G  G  D  P  C  N  P  F  V  *  F  C  -
       L  C  A  P  R  N  G  E  E  G  T  H  V  I  L  L  F  S  F  V  -
```

FIG. 2(x)



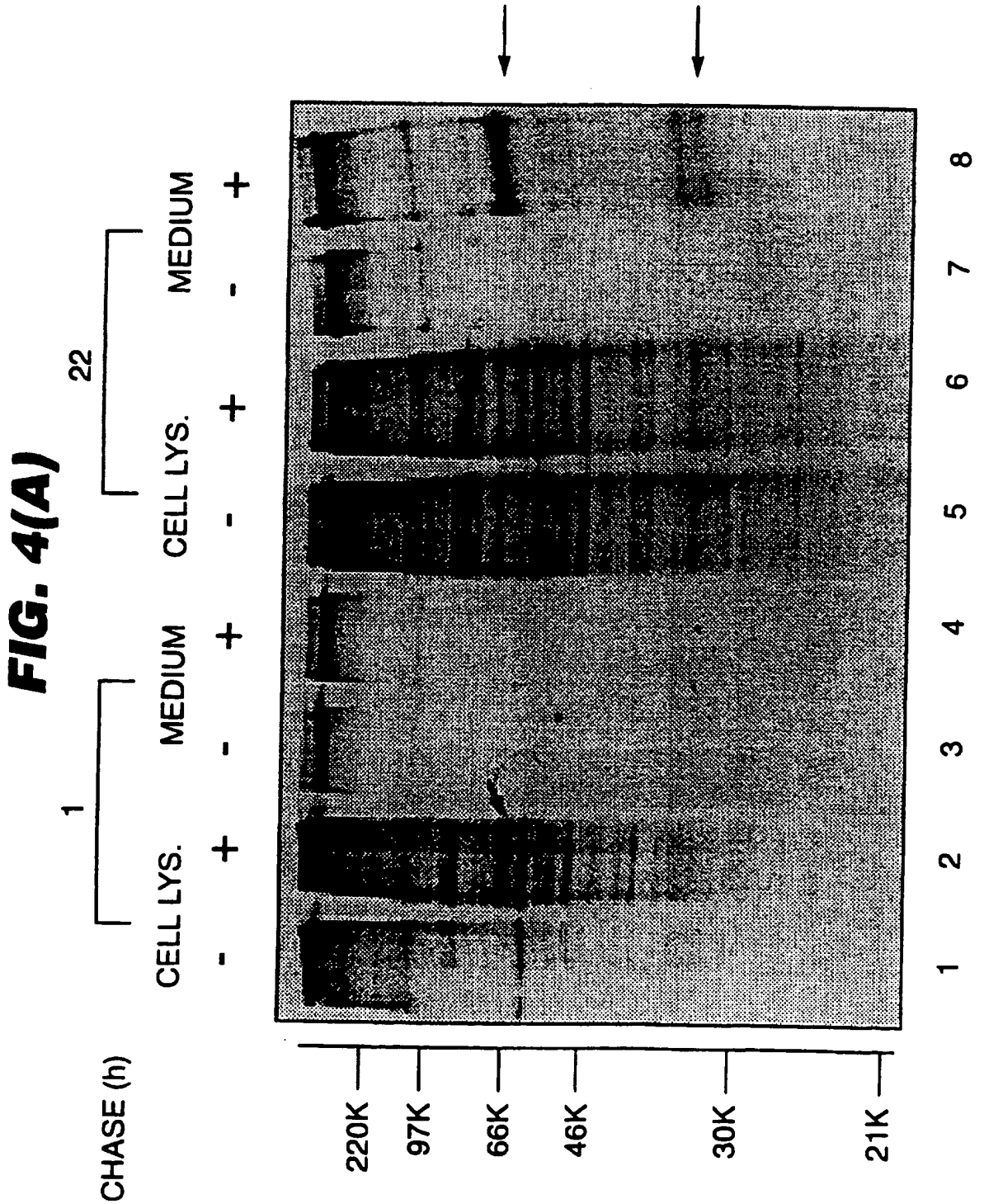
```
                                                                    TTCTGATTACTGTCCAGCTTATAGTCTTCCAGTTTAATGAACTACCATCTGATGTTTCAT
1741 ----------+----------+----------+----------+----------+----------+----------+ 1800
                AAGACTAATGACAGGTCGAATATCAGAAGGTCAAATTACTTGATGGTAGACTACAAAGTA

                F  *  L  L  S  S  L  *  S  S  S  L  M  N  Y  H  L  M  F  H  -
                S  D  Y  C  P  A  Y  S  L  P  V  *  *  T  T  I  *  C  F  I  -
                L  I  T  V  Q  L  I  V  F  Q  F  N  E  L  P  S  D  V  S  Y  -

                ATTTAAGTGTATTTAAAGAAATAAACACCATTATTCAAGCCATATAAAAAAAAAAAA
1801 ----------+----------+----------+----------+----------+----------+----------+ 1860
                TAAATTCACATAAATTTCTTTATTTGTGGTAATAAGTTCGGTATATTTTTTTTTTT

                I  *  V  Y  L  K  K  I  N  T  I  I  Q  A  I  *  K  K  K  K  -
                F  K  C  I  *  R  K  *  T  P  L  F  K  P  Y  K  K  K  K  K  -
                L  S  V  F  K  E  N  K  H  H  Y  S  S  H  I  K  K  K  K  K  -

                AAAA
```

FIG. 2(XI)

FIG. 3

```
FDGF     (103) TLKVIDEEWQ RTQCSPRETC VEVASELGKT T..NTFFKPP •CVNVFRCGGC
VEGF-C   (49)  ILKSIDNEWR KTQCMPREVC IDVGKEFGVA T..NTFFKPP CVSVYRCGGC
VEGF     (39)  EVVKFMDVYQ RSYCHPIETL VDIFQEYPDE I..EYIFKPS CVPLMRCGGC
PlGF     (39)  EVVPFQEVWG RSYCRALERL VDVVSEYPSE V..EHMFSPS CVSLLRCTGC
PDGF-B   (84)  GSLTIAEPAM IAECKTRTEV FEISRRLIDR TNANFLVWPP CVEVQRCSGC
PDGF-A   (83)  RRKRSIEEAV PAVCKTRTVI YEIPRSQVDP TSANFLIWPP CVEVKRCTGC

FDGF     (151) •CNEEGVMCMN TSTSYISKQL FEIS.VPLTS VPELVPVKIA •NHTGCKCLPT
VEGF-C   (97)  CNSEGLQCMN TSTSYLSKTL FEIT.VPLSQ GPKPVTISFA NHTSCRCMSK
VEGF     (87)  CNDEGLECVP TEESNITMQI MRIK..P.HQ GQHIGEMSFL QHNKCECRPK
PlGF     (87)  CGDENLHCVP VETANVTMQL LKIR..S.GD RPSYVELTFS QHVRCECRPL
PDGF-B   (134) CNNRNVQCRP TQVQLRPVQV RKIEIVRKKP IFKKATVTLE DHLACKCETV
PDGF-A   (133) CNTSSVKCQP SRVHHRSVKV AKVEYVRKKP KLKEVQVRLE EHLECACTT
```

FIG. 4(A)


CHASE (h)
1
22
CELL LYS.
MEDIUM
CELL LYS.
MEDIUM
- + - + - + - +
220K
97K
66K
46K
30K
21K
1 2 3 4 5 6 7 8

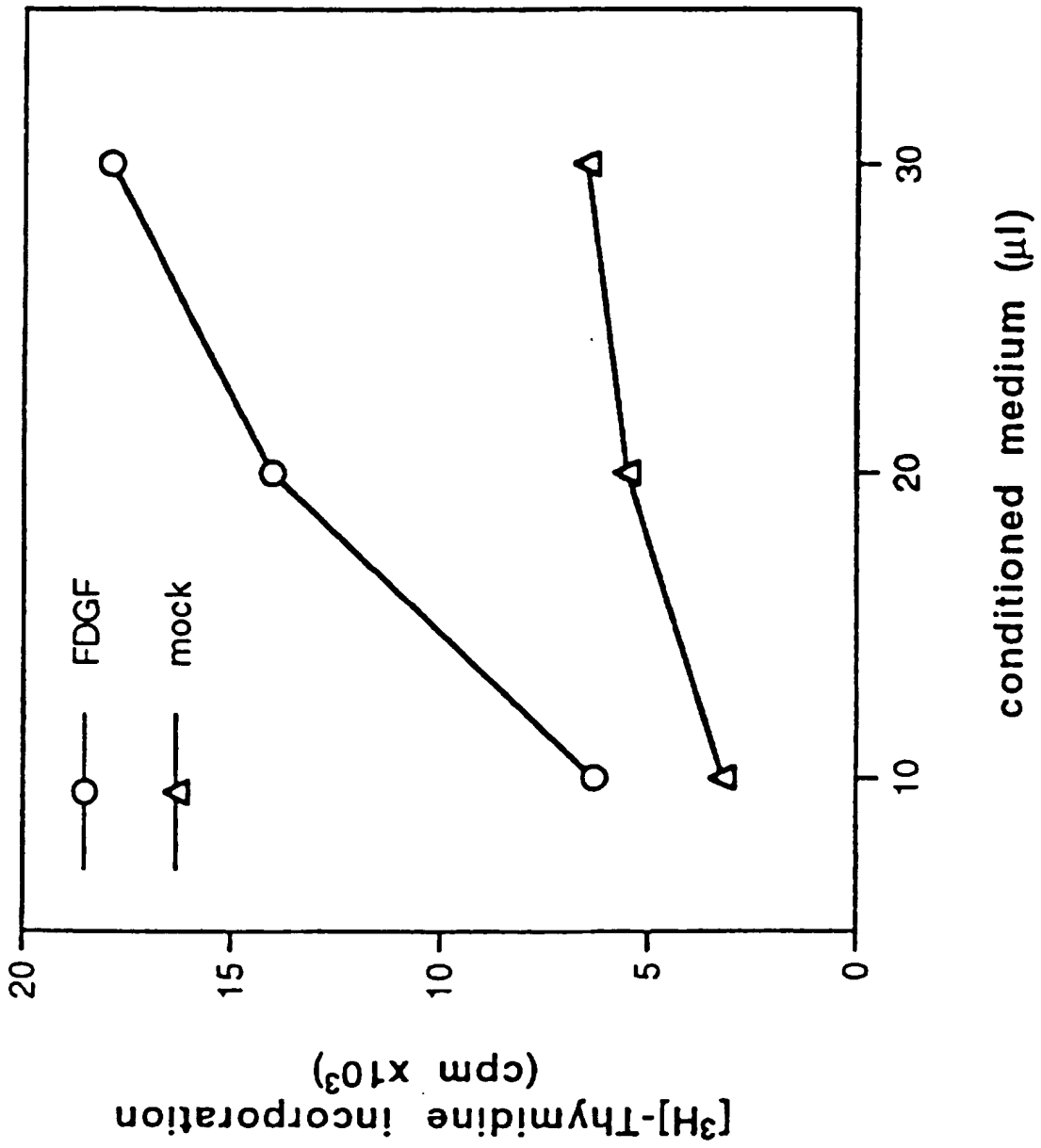

FDGF
mock
20
15
10
5
0
10
20
30
conditioned medium (µl)
[3H]-Thymidine incorporation
(cpm x10³)

FIG. 4(B)

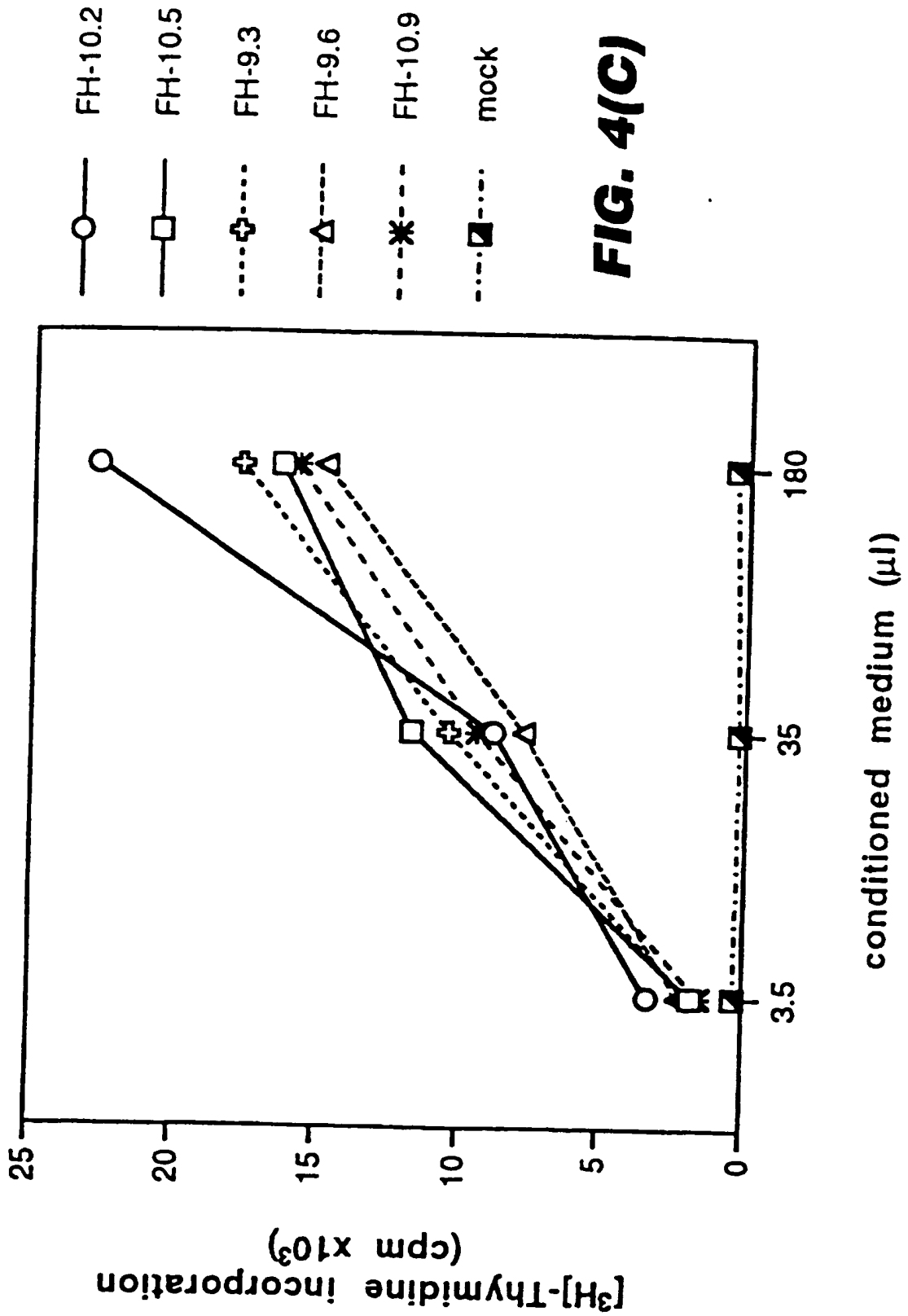

FH-10.2
FH-10.5
FH-9.3
FH-9.6
FH-10.9
mock
[3H]-Thymidine incorporation (cpm x10³)
0
5
10
15
20
25
3.5
35
180
conditioned medium (µl)

FIG. 4(C)

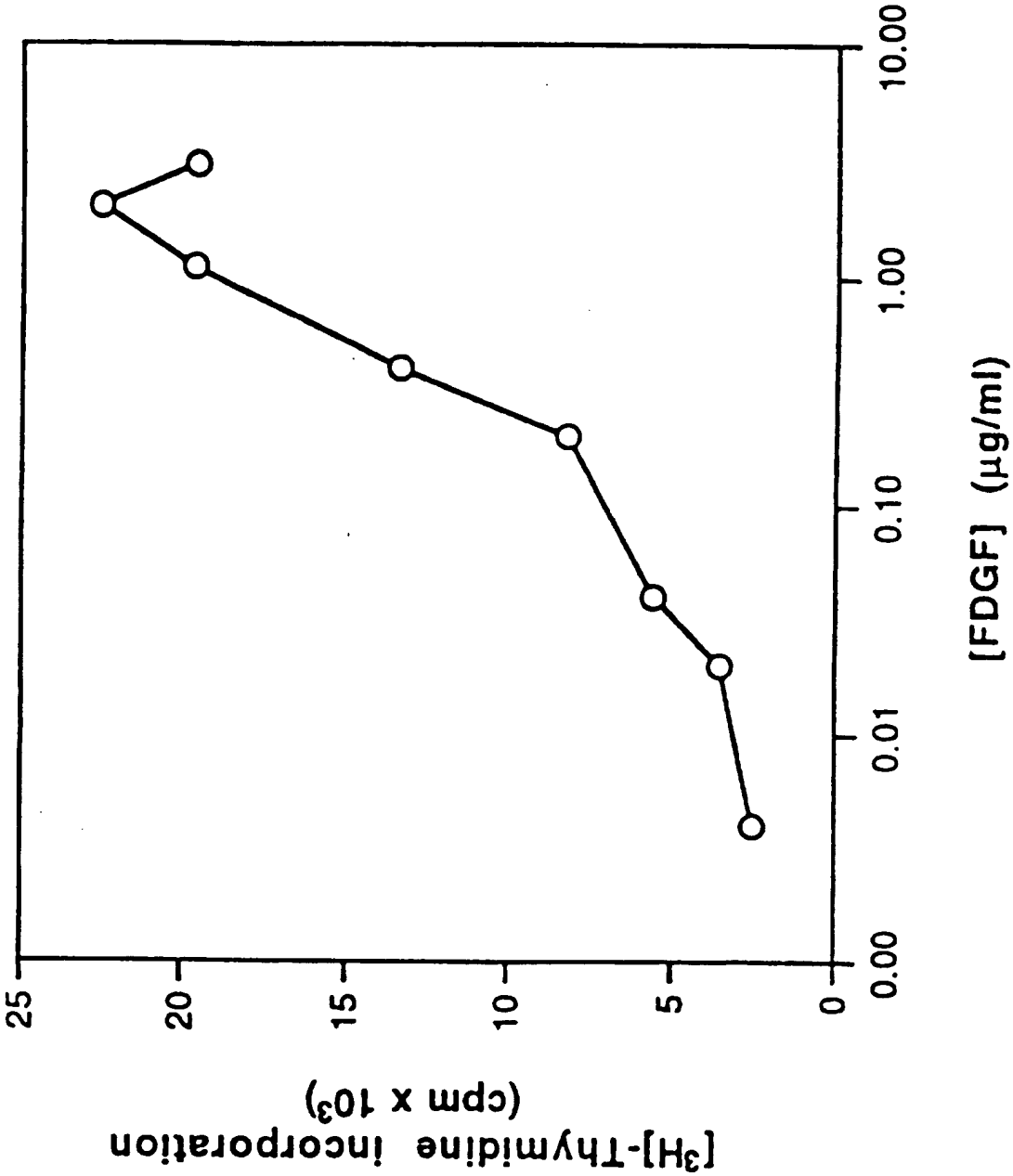

[FDGF] (µg/ml)
0.00
0.01
0.10
1.00
10.00
[3H]-Thymidine incorporation
(cpm x 10³)
0
5
10
15
20
25

FIG. 4(D)

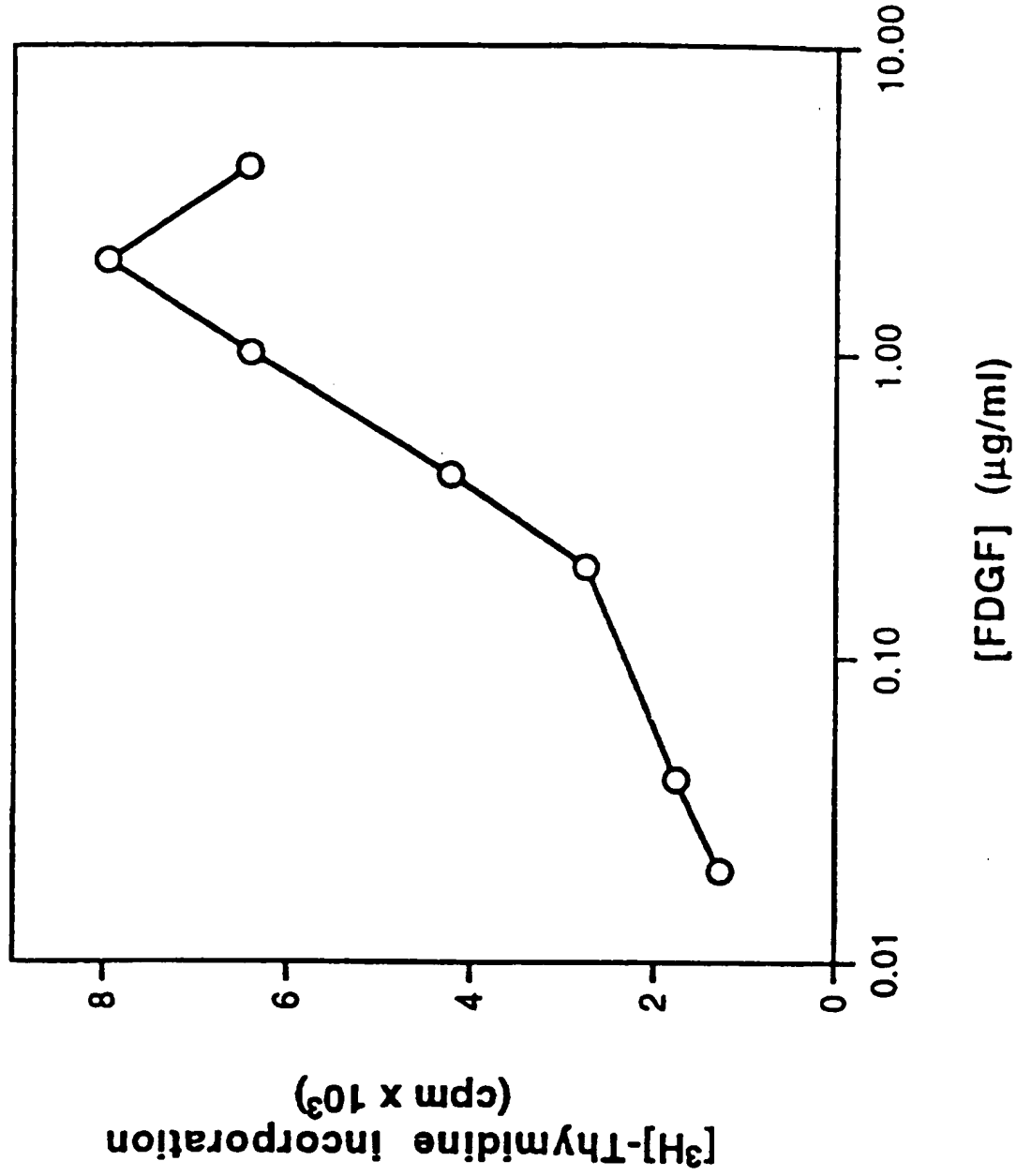

[FDGF] (µg/ml)
0.01
0.10
1.00
10.00
0
2
4
6
8
[3H]-Thymidine incorporation
(cpm x 10³)

FIG. 4(E)

FIG. 5(A)

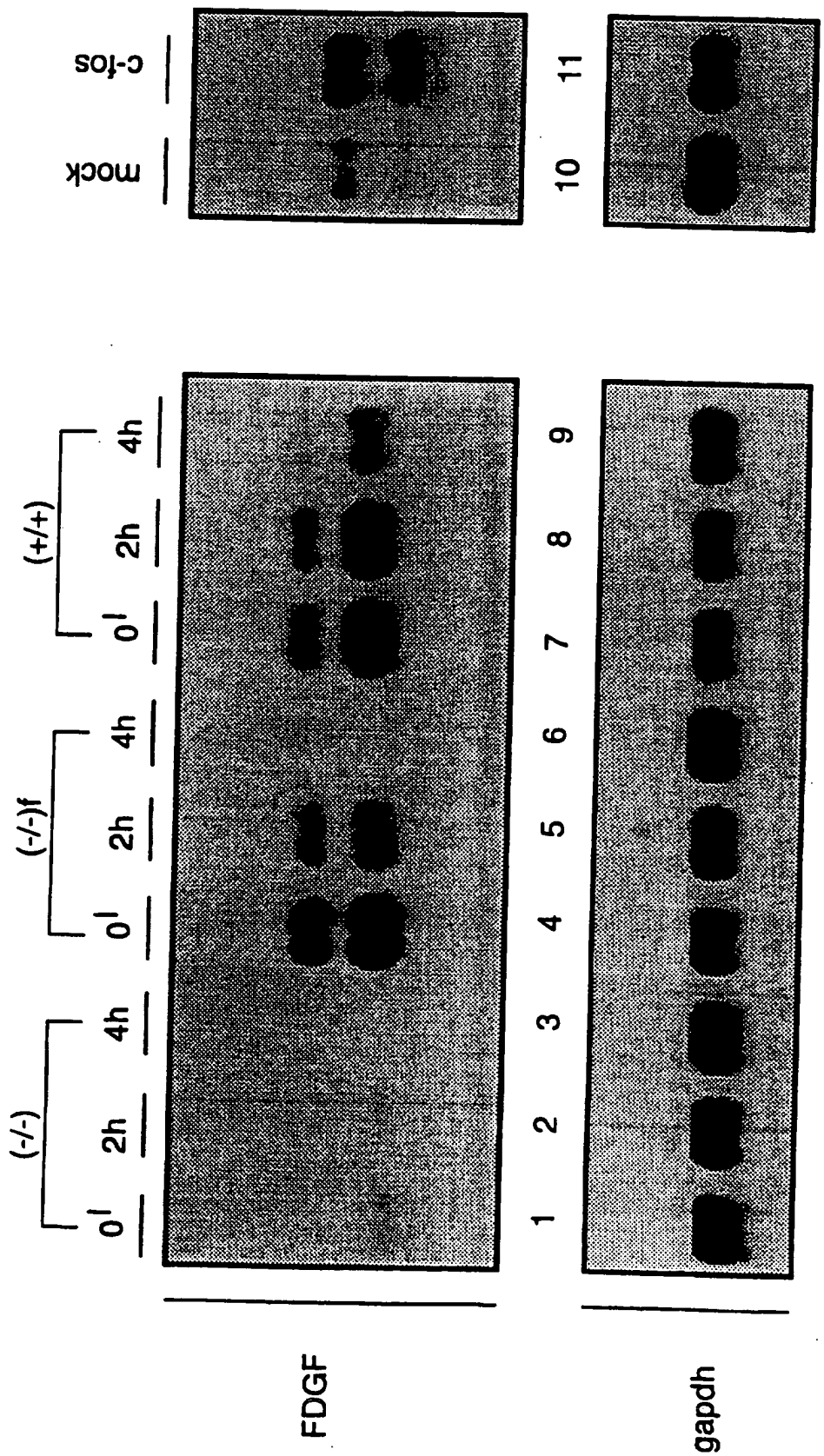


FIG. 5(B)

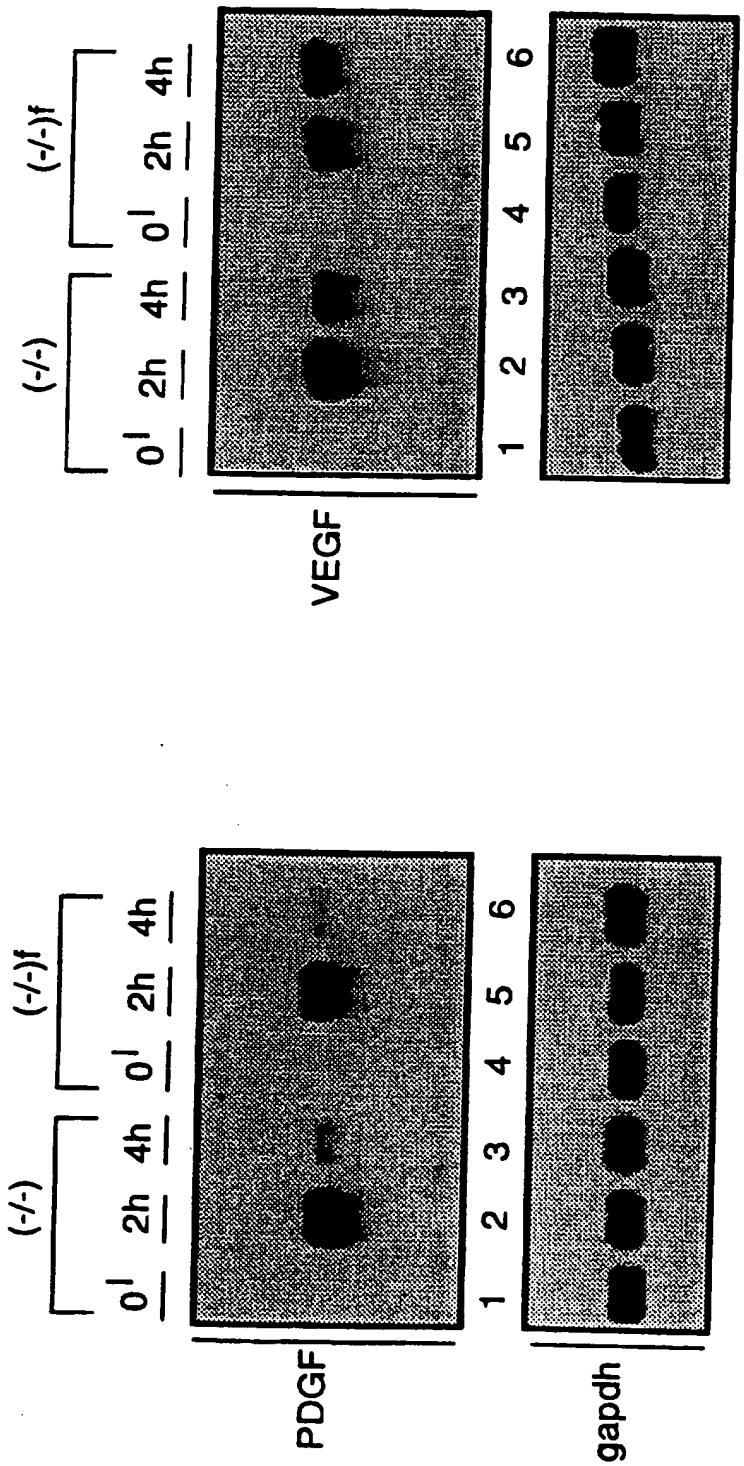


FIG. 6

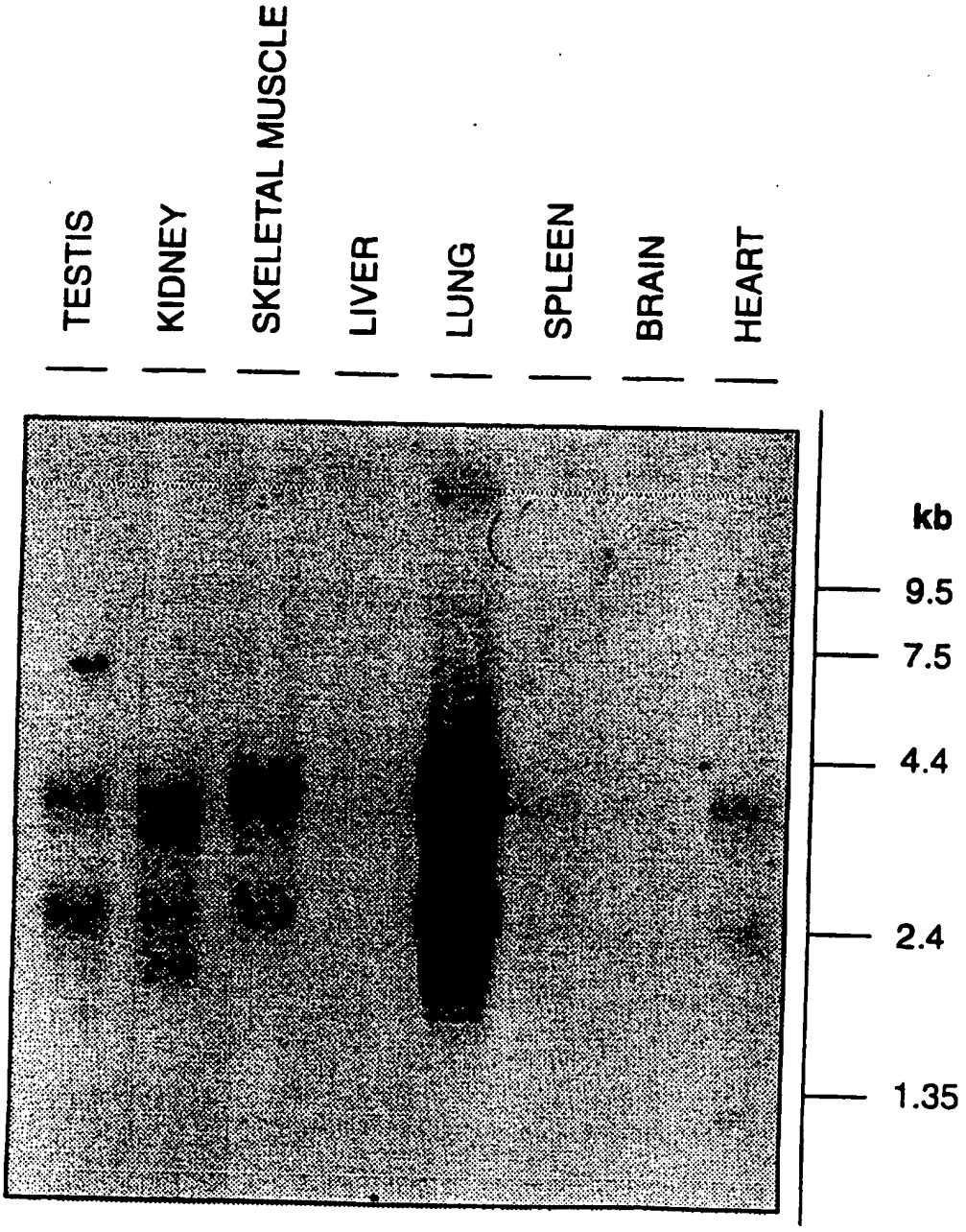

TESTIS
KIDNEY
SKELETAL MUSCLE
LIVER
LUNG
SPLEEN
BRAIN
HEART
kb
9.5
7.5
4.4
2.4
1.35

FIG. 7(C)

FIG. 7(B)

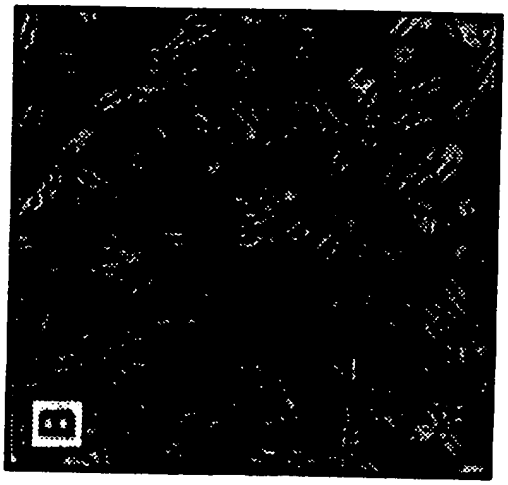


FIG. 7(A)

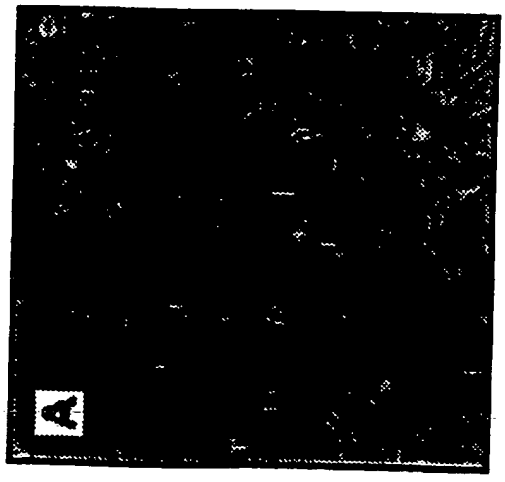


FIG. 7(F)

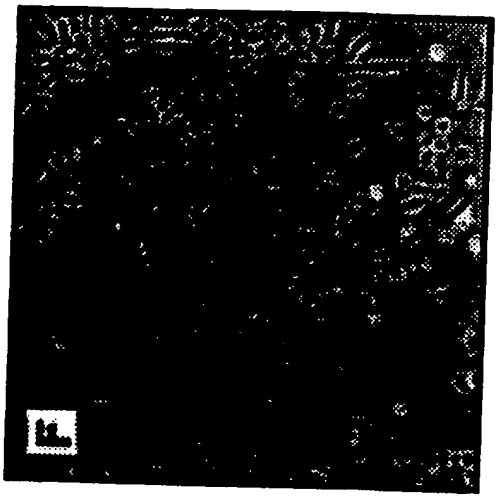


FIG. 7(E)

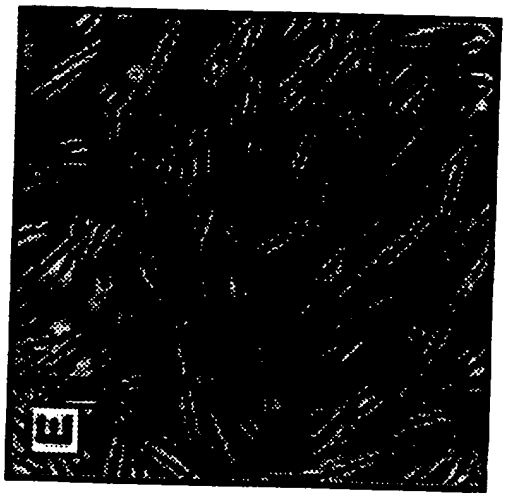


FIG. 7(D)

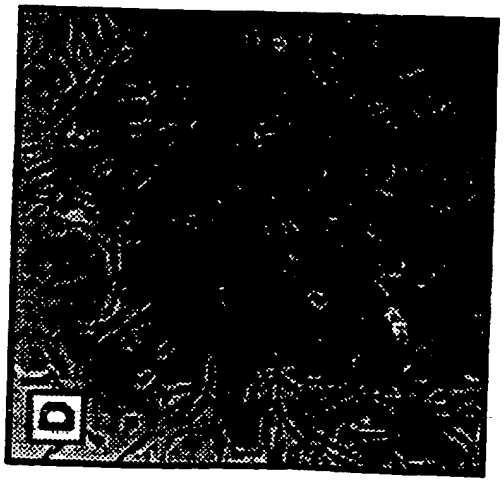


FIG. 7(G)

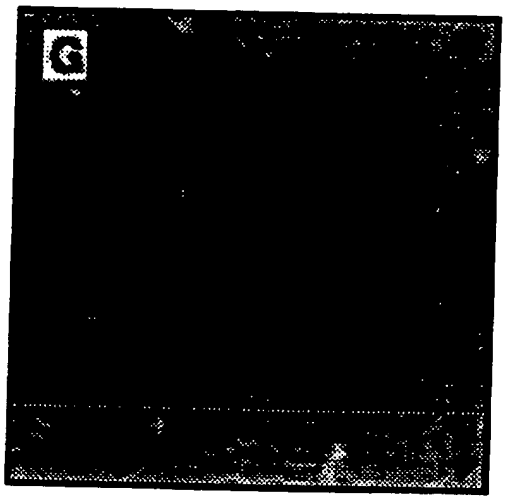


FIG. 7(H)